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Physiological Zoölogy

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IS FOOD THE EFFECTIVE GROWTH-PROMOTING FACTOR IN HOMOTYPICALLY CONDITIONED WATER?[1]

(One plate)

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THIS is a progress report concerning the results obtained to date in a continuing series of experiments upon mass physiology as a factor in the growth of fishes. The publication of this analysis at this time is justified by the fact that we have arrived at definite conclusions with regard to certain fundamental aspects of the problems involved and by the mutually regretted withdrawal of one of the junior authors (Dr. R. B. Oesting) from active participation in the investigation.

Earlier studies have shown that, other things being equal, and under the conditions of our experiments, isolated goldfishes grow more rapidly if placed in water in which other goldfishes have lived than if in uncontaminated but otherwise similar water. In connection with tests in distilled water, a synthetic pond water was developed in which the total electrolytes were not changed by exposure to fishes for 22 hours under the usual conditions of assay. This synthetic pond water contained 100 mg. $CaCl_2$ and 50 mg. each of $NaNO_3$, $MgSO_4$, and K_2SO_4 per liter of high-grade distilled water

[1] We are indebted to Grassyforks Fisheries, Inc., Martinsville, Indiana, for the gift of the goldfishes used in these experiments; and to Miss Gertrude Evans and Mr. Wayne Livengood for access to their unpublished studies on the growth of fishes. This generous co-operation is thoroughly appreciated.

(Allee, Bowen, Welty, and Oesting, 1934). This same formula for synthetic pond water has been used in the present experiments; tests of other waters are in progress.

In general and except as stated, the experimental procedures described in the paper cited above have been followed in the experiments now to be reported, except that in later work glazed earthenware jars holding approximately 4 liters have been substituted for glass aquaria. Throughout the work the commonest variety of single-tailed goldfishes, *Carassius auratus*, has been used exclusively. In recent experiments all have been supplied by the same hatchery.

Including results hitherto published, we have to date a series of twenty-seven paired experiments in which the growth of fishes isolated into untreated conditioned water is compared with that of fishes similarly isolated into comparable, though biologically uncontaminated, control water. In all we have records (through H7) from 290 experimental and 274 control fishes that lived through the 20–30 days of assay for this experiment alone. With all of these, the full conditioned water, not sieved or filtered, was used to test its effect on growth; faeces, slime, and regurgitated food particles were all present indiscriminately as they were introduced by the conditioning fishes. It is worth noting specifically that the conditioning fishes were fed daily in water other than that which they were conditioning. After 2 hours of feeding they were washed until traces of food were removed from their bodies and until the first regurgitations, if any, were also removed, and were finally rinsed briefly in distilled water before being again used as conditioning fishes for the following day.

Growth was determined by the photographic method described in the report cited above. Photographs were taken under standard conditions at the beginning and at the end of the assay period. These were measured to determine growth in length from anterior margin of the eye to the base of the tail, and also at the region of greatest width. All photographs are on file. Since the photographs are slightly magnified, the growth as recorded is to be regarded as in artificial units which must be multiplied by 0.82 to obtain the actual growth of the fishes in millimeters. Critical measurements in the present report are in length and have an accuracy of ± 0.04 per cent,

which makes the actual error for the fishes used approximately ± 0.02 units.

For the growth experiments with full-conditioned and otherwise untreated conditioned water, there was a mean growth of 1.8 units for the experimental fishes and of -0.23 units for the accompanying controls. Each of the twenty-seven available experiments has the mean difference in the same direction, and in all but three of the individual experiments the results are statistically significant.[2] Taken as a whole, the mean difference in growth of 2.03 units has $P = < 0.0001$, which means that there is less than one chance in ten thousand of obtaining similar results by random sampling.

As stated in the previous report, in an attempt to eliminate grosser particles of faeces and regurgitated food, the conditioned water was strained through a phospho-bronze screen with 325 meshes per inch; the openings average 0.040 mm. The control water was similarly strained through another and wholly similar sieve. We now have a total of fifteen paired experiments involving in all 180 fishes assayed in the sieved conditioned water and 142 accompanying controls. The former grew 1.85 units in length; the latter, 1.00 unit. The mean difference of 0.85 has a P-value of < 0.0001 and is statistically significant.

These two sets of experiments have been made with the fishes in conditioned water, all of which had been exposed to the conditioning fishes. We have continued to gain experience with the use of a smaller amount of the conditioning water, which became much more highly charged with organic materials obtained from the contained fishes. A certain amount of this water, usually 190 cc. per 2 liters, was added to water similar to that in the controls to make the conditioned water used in the growth assays. We now have data from fifteen such experiments with 210 experimental and 130 control fishes. The fishes in the conditioned water grew 2.28 units; those from the accompanying controls grew 1.28 units. The difference of 1.00 has a P value of < 0.0001.

These three types of experiments extend and confirm the findings

[2] In this paper statistical significance is given in terms of P and is based on "Student's" method for finding statistical significance of relatively small numbers of paired observations. $P = 0.05$ is equivalent to three times the probable error; this value is taken as the extreme upper limit of statistical significance.

already reported. The additional data on these points have been accumulated incidentally as normal or conditioned water controls while prosecuting analysis of the nature of the conditioning factor, some of the results of which will be presented in the following pages; others will be reported elsewhere. There is no doubt but that under the conditions of our experiments goldfishes of about an inch in body length grow faster in 20–30 days of assay when in freshly prepared and daily changed homotypically conditioned water than when in similar but biologically uncontaminated water (*a*) when nothing is removed from the water, (*b*) when particles larger than 40 μ are screened out, and (*c*) when a small amount of water is heavily conditioned and screened and then diluted to the usual conditioning strength.

We have some evidence which indicates that there is less difference in growth of fishes in homotypically conditioned water, as compared with that of accompanying control fishes, when the latter show extra-good or superb growth during the assay period. In part, at least, this seems to be a matter of the amount and kind of food given the assay fishes. Further and more exact discussion of this problem is reserved for the present.

Effects of different amounts of conditioning.—In the general work on the effect of biological conditioning of the water on various biological processes and in early work on the present problem, it was found to be comparatively easy to demonstrate that overconditioning produces harmful effects (Allee, 1931, 1934); in terms of the present experiments, such overconditioning retards, rather than promotes, growth. The retarding effect is usually attributed to the effect of the presence of metabolic wastes or to decomposition products of these. This immediately raises the question concerning whether the amount of conditioning used in these experiments was above or below the optimal amount.

The amount of conditioning used here was in all cases that which could be accomplished in about 22 hours. No food was added directly to the water being conditioned, since the conditioning fishes were fed in other water during the two remaining hours of the twenty-four. The amount of conditioning is approximately a factor of the number and the size of fishes present, together with the volume

of the water to which they were exposed, and may be indicated by a "conditioning coefficient." This coefficient is obtained by dividing the product of the number of conditioning fishes times their length in millimeters by the number of liters of water conditioned. In the experiments summarized in the preceding pages this coefficient varied from 12 to 74; 45 of the 58 lots of water had a conditioning coefficient of between 20 and 30; 6 were over 40; and the highest three were 49, 52, and 74, respectively. For example, in Experiment 7 there were approximately 0.165 fishes 76 mm. long per liter of water; and in Experiment 19 there were 0.95 fishes 47 mm. long per liter for the same amount of water. The former gives a conditioning coefficient of 12.5; the latter, one of 44.7.

When the amount of growth in any given experiment is plotted against the conditioning coefficient, it was found that in these experiments 24 is about the median value. Such a plotting reveals a wide variation in growth-rate at any given level of conditioning, as might be expected considering the number of known variables. The mean growth in the twenty-three experiments below the median conditioning coefficient was 1.57 units; for the eight experiments at the median, the mean growth was 2.11 units; for the twenty-six experiments above this coefficient, it was 2.17. (The exact conditioning coefficient of one experiment is not now known.) These results indicate that we have not been overconditioning the medium, at least in the majority of the experiments; instead there has been too little, rather than too much, conditioning for most rapid growth.

One experiment (H-7) was devised in part to test the effect of different amounts of conditioning upon the rate of growth. The results obtained are summarized in Table I. The same concentrated conditioned water was used in all, but the proportions added to make up the assay volume of 2 liters was varied to give the conditioning coefficients of 25, 49, and 74, respectively. Under these conditions there was the most rapid growth at the intermediate strength used, which was significantly greater than the growth given with the most strongly conditioned water.

This direct test supports the conclusions reached from a study of the growth in relation to the amount of conditioning in the other ex-

periments; certainly, in general, we have been working below, rather than over, the conditioning value which gives the most rapid growth under the conditions of these experiments. The exact optimum amount of conditioning has not been established.

The fed conditioning fishes were given a varied and adequate diet, which, with one exception, contained either *Daphnia* or fresh liver or both. Although the effect of the diet fed the conditioning fishes deserves direct experimental analysis, this has not been made as yet, beyond the tests to be reported later in the present paper, in which the conditioning fishes were starved. When the water was conditioned by starved fishes, the animals to be used in a given experiment were starved for 5 days before the experiment was begun,

TABLE I

THE EFFECT OF DIFFERENT AMOUNTS OF CONDITIONING UPON RATE OF GROWTH IN GOLDFISHES IN AN ASSAY PERIOD OF 20 DAYS

Columns	Observed Growth			Statistical Probabilities		
	i	ii	iii	ii−i	ii−iii	i−iii
Conditioning coefficients	25	49	74	.....	.....	.....
Growth in length	1.5	1.9	1.2	0.19	0.03	0.32
Growth in length plus width	1.7	2.4	1.4	0.13	0.045	0.48

and were used as conditioning fishes for a 10-day period, when a similarly prepared lot were substituted. In the first of these tests, the same lot of fasting fishes was used throughout.

There are several lines of evidence that food materials are present in the conditioned water whether screened or unscreened. In the first place, such water is opalescent and contains strands of mucus and other suspended particles visible to the eye. The appearance of the water changes when the conditioning fishes are fed liver, as compared with rolled oats. Faeces are present in the unscreened water in relatively large pieces. These are ingested at times, as shown by the following observations: Two fishes, starved for 4 days, were placed in 500 cc. of synthetic pond water which contained four pieces of faeces on which flecks of charcoal had been carefully placed. The next morning there was but one piece of faeces in the container, and

this one had charcoal on the inside of the coil and was coated on the outside with the usual jelly-like characteristic of goldfish faeces.

Two other fishes starved for 4 days were placed in the same amount of synthetic pond water which contained suspended charcoal that had passed through one of the 325-mesh phospho-bronze sieves in regular use for straining this water. The faeces of these fishes contained charcoal the following morning; this shows that particles may be ingested even when they are 40 μ or less in diameter. This experiment has been repeated twice, once with identical results and again when the fishes yielded either no faeces or clear jelly-like strands only.

Attempts to assay the food value of conditioned water include (*a*) measurement of required oxygen; (*b*) spectrographic analysis; (*c*) conditioning by fasting fishes; (*d*) filtering the conditioned water; (*e*) comparison with results obtained by the addition of minute particles of food in direct conditioning, and (*f*) assays to determine possible vitamin effects from the fish-conditioned water. Results from (*e*) and (*f*) will be reported elsewhere. It is pertinent here to inquire whether the observed increase in growth of fishes in conditioned water is caused wholly or in part by increased food intake.

Required oxygen.—Required oxygen measures the total amount of oxygen necessary to oxidize the organic material present to a fairly stable end-product. This gives a measure of the total amount of oxidizable organic matter present, but does not, of course, measure its availability as food. The method used was that described in *Official and Tentative Methods of Analysis*, of the Association of Official Agricultural Chemists (1925), p. 87. Results are given in milligrams of oxygen consumed per liter when the period of boiling with permanganate is limited to 10 minutes. Tests were usually made at ten times the assay strength; these give results similar to those in H-4, in which the tests were made on the assay-strength water. In the latter experiment the observed differences were too slight to be measured with certainty by this method, but in each case they were in the same direction as were the similar differences obtained in the other experiments. The data for the required oxygen and for growth in the several types of water tested are summarized in Table II.

STATISTICAL PROBABILITIES

V–IX, $P=0.0016$ IV–XII, $P=0.9$
VI–X, $P=0.0094$ VIII–XII, $P=1.0-$
VI–XII, $P=<0.0001$ IV–X, $P=0.558$
X–XII, $P=0.0134$

Study of this table shows that the controls growing in water which at no time absorbed oxygen grew, on the average, 1.55 units. Sig-

TABLE II

REQUIRED OXYGEN IN FISH-CONDITIONED WATER IN RELATION TO FISH GROWTH IN SUCH WATER GIVEN IN TERMS OF STRENGTH CALCULATED FOR THE WATERS TO WHICH THE FISHES WERE EXPOSED

Experiment No.	Conditioning Coefficient	Conditioning Fishes—Fed				Conditioning Fishes—Starved				Control	
		Filtered		Sieved		Filtered		Sieved			
		Required Oxygen (Mg.)	Growth in Length (Mm.)	Required Oxygen (Mg.)	Growth in Length (Mm.)	Required Oxygen (Mg.)	Growth in Length (Mm.)	Required Oxygen (Mg.)	Growth in Length (Mm.)	Required Oxygen (Mg.)	Growth in Length (Mm.)
i	ii	iii	iv	v	vi	vii	viii	ix	x	xi	xii
H-1	13	.....	.....	0.16	4.22	.....	.....	0.08	3.86	0.0	3.6
H-2	24	0.28	3.70	0.35	5.10	0.08	4.00	0.08	4.80	0.0	4.0
H-3	26	0.43	1.66	0.51	1.44	0.13	1.54	0.13	1.53	0.0	1.1
H-3	26	0.43	1.14	0.51	1.52	0.13	1.23	0.13	1.84	0.0	1.1
H-4	26	0.44	4.27	0.65	4.76	0.09	3.30	0.16	3.80	0.0	4.2
H-5	27	0.72	2.10	0.80	2.10	0.15	1.40	0.18	1.20	0.0	1.1
H-6	27	0.64	1.00	0.74	2.00	.....	.....	0.25	0.90	0.0	0.7
H-6	26	.....	.....	1.16	1.40	.....	.....	.....	.....	0.0	0.8
H-6	52	.....	.....	2.32	1.60	.....	.....	0.50	0.90	0.0	0.96
H-7	25	.....	.....	0.62	1.50	.....	.....	0.15	0.60	0.0	0.3
H-7	49	.....	.....	1.24	1.90	.....	.....	0.30	0.70	0.0	0.7
H-7	74	.....	.....	1.88	1.20	.....	.....	0.45	1.00	0.0	0.0
Mean	.....	0.49	2.31	0.91	2.40	0.12	2.29	0.22	1.92	0.0	1.55

nificantly increased rates of growth above this level must be correlated with some aspect of the conditioning effect. The differences in growth in filtered water, whether from fed or from starved conditioning fishes, is not significant; but there are too few cases recorded in this table for this to be taken as a final result. The fishes in water from starved and from fed conditioning fishes which had been sieved, rather than filtered, grew significantly more than did those in the

controls; and those in water conditioned by fed fishes grew more than those from the water conditioned by starved fishes. The recorded differences for these fishes are statistically significant both as regards growth and differences in required oxygen; the greater the mean amount of required oxygen, the more growth. Stating this in other terms, the greater the possible food value of the conditioned water, the greater the growth, except when filtered conditioned water was tested; and in the few tests recorded here these filtered waters did not promote growth significantly better than did the control water, even though one set required double the amount of oxygen, as did the sieved water from starved conditioned fishes, which did stimulate growth significantly.

In order to test the matter further, the correlation coefficient was calculated for the amount of increase in growth of the experimental fishes in sieved conditioned water, whether from fed or from starved fishes, over that in the accompanying controls. There is a slight, but apparently trustworthy, positive correlation, with $r = 0.066 \pm$ a standard error of 0.021.

Conditioning by fasting fishes.—Another method of testing whether the beneficial effects of conditioned water of the strength used resulted from the addition of food values by the conditioning fishes is to starve the fishes used to condition the water. As already stated, such conditioning fishes were starved for 5 days before the experiment began, and after the first trial were replaced at the end of each 10-day period by other fishes that had also been starved for a preliminary period of 5 days. This change was made because of the possibility that the fasting fishes might well cease producing as much slime, or other conditioning factors other than food, as would normally fed fishes. This water conditioned by fasting fishes was screened as usual, and the experiments were conducted in all details according to standard practise. The conditioning coefficient was 27 or less in ten of the twelve comparable experiments and went above 70 in the most highly conditioned one.

It is probably important in this connection that, while with fed conditioning fishes this highly conditioned water was definitely more harmful than was water with a conditioning coefficient of about 50, with these starved fishes the growth was decidedly better than with

the lower concentrations and was, in fact, the only experiment of this series in which there was statistically significant growth in any one experiment considered alone.

The data for these experiments are summarized in Table II, which was presented in another connection in an earlier section. This table gives the results from 161 experimental and 114 control fishes. Despite the lack of statistical significance in any individual experiment except when the most highly conditioned water was used, when all are considered as twelve paired experiments the difference in growth of 0.37 units (columns x–xii in Table II) has a statistical probability of 0.0134, which must be taken seriously.

If the food introduced by conditioning fishes is the principal factor in promoting growth in homotypically conditioned water, then, other conditions being equal, water in which fasting fishes have lived should have less growth-promoting power than if it is conditioned by fed fishes. The experiments summarized in columns vi and x of Table II give the available data on this point; in all of these, comparable fishes were assayed simultaneously in the two types of conditioned waters. Because of differences in growth-rates at different times and because of possible variations in environmental conditions in the laboratory, it is not safe to rely for critical results on growth differences between groups tested at different times. For the slight changes with which we are dealing, paired experiments are much safer.

With the eleven paired experiments at hand, there was greater mean growth of 0.48 units in the water conditioned by fed fishes. The statistical probability is 0.0094. In these experiments, placing assay fishes in water conditioned by fasting fishes increased their growth-rate during the assay period slightly but significantly. The use of water conditioned by fed fishes caused a somewhat greater and somewhat more significant increase in growth over and above that produced by the water from fasting fishes.

It is significant for our problem to note that an increase of required oxygen from 0.0 to 0.22 cc. per liter over the untreated controls produced almost as great a mean increase in growth as did an increase from 0.22 to 0.91 cc. of required oxygen per liter. Either the conditioning fishes introduce some growth-promoting substance other

than food into the water or the amount of growth is relatively much more stimulated by the presence of a very slight initial amount of food material than it is by further and more marked increases. This problem must be carried forward into the next section.

Filtered conditioned water.—Another method of removing the possible food value of the conditioned water is by filtering. The effect of this treatment on growth is summarized in Table III, which records

TABLE III

SHOWING THE EFFECT OF FILTERING OF CONDITIONED WATER ON GROWTH; IN O THROUGH ONE No. 40 WHATMAN PAPER, IN H THROUGH TWO S. AND S. No. 595 PAPERS

Experiment No.	Days Tested	Conditioned by Fasting Fishes		Control		*P*	Conditioning Coefficient	Conditioned by Fed Fishes	
		No. of Fishes	Growth (Mm.)	Growth (Mm.)	No. of Fishes			No. of Fishes	Growth (Mm.)
O-11E	30	10	4.5	3.1	9	None	24	.....	.....
O-11D	30	10	5.9	3.7	10	0.007	24	9	3.1
O-11k	30	10	4.7	3.6	10	None	24	.....	.....
O-11m	30	10	4.4	3.3	10	None	24	.....	.....
O-12AB	20	7	0.06	−0.03	7	None	17	.....	.....
O-12eD	20	7	0.0	0.7	5	None	17	.....	.....
O-12Lk	20	10	0.7	0.6	5	None	17	.....	.....
O-12nm	20	5	0.2	0.3	8	None	17	.....	.....
H-2	30	15	4.0	3.9	15	None	24	15	3.70
H-3	20	9	1.54	1.1	10	None	26	10	1.66
H-3	20	9	1.23	1.1	10	None	26	10	1.14
H-4	20	9	3.3	4.2	9	None	26	10	4.27
H-5	20	15	1.4	1.1	15	None	26	15	2.10
Total (13)	310	126	2.46	2.0	123	0.0816	.....	69	2.66

the results from thirteen experiments with filtered conditioned water from fasting fishes and of six experiments with similarly filtered water from fed conditioning fishes which were made simultaneously with some of the experiments on water from fasting fishes. In the experiments marked "O" the water was filtered through one thickness of Whatman No. 40 filter paper; in those marked "H," through two thicknesses of No. 595 Schleicher and Schull paper.

Required oxygen was not determined for all these experiments. Turning again to Table II, one finds that, for those determinations

made, the filtered water from fasting fishes required 0.12 mg. oxygen per liter and the filtered water from fed conditioning fishes required approximately four times as much. Neither grew significantly more than did the controls, and in the paired experiments the two lots grew almost exactly the same whether in one or the other filtered conditioned water. When all the experiments from filtered water from fasting conditioning fishes are considered, the data summarized in Table III show that the observed increase in growth in the conditioned water of 0.46 units approaches, but by no means reaches, statistical significance.

Other experiments are available on the effect of filtering water from fed conditioning fishes which were not made at the same time as those summarized in Table III. When all these are considered with their respective controls as one series of twenty-one paired experiments, there is a mean increase in growth of 0.36 units, with $P = 0.007$ and hence significant statistically.

The results obtained with these filtered conditioned waters can be explained if one assumes either that the filtering removes some growth-promoting substance or that it takes out the food particles which are of sufficient size to be of value as fish food although it does not remove all the organic matter. According to the required-oxygen data (Table II), filtering removes about half of the organic material from the water, whether it comes from fed or from fasting conditioning fishes. We are not yet in a position to decide with certainty between these two possibilities, which will be discussed again later in the present report.

Protein extract.—If food is the primary growth-promoting factor in conditioned water, exceedingly small amounts of extract from the skin of goldfishes would not be expected to have an effect in stimulating growth. On the other hand, if there is some sort of growth stimulant, other than food, in the homotypically conditioned water, one of the possible sources would be found in the secretions given off from the surface of the fishes. This offers a new and attractive avenue of approach to the whole problem, which has been and is being investigated.

A protein extract of the surface of goldfishes was prepared as follows: Fifteen goldfishes, averaging 50 mm. from tip to origin of tail

fin, were placed in an acetate buffer at pH 4.15–4.20. At this pH the slime coating on the bodies was precipitated as a white material, which was scraped off, washed, and then dissolved in 10 cc. of N/2 NaOH plus 25 cc. distilled water. The solution was filtered and adjusted to pH 4.2 with N/2 H_2SO_4. The resulting protein precipitate was washed by decantation and dissolved in 10 cc. of N/2 NaOH; enough N/2 H_2SO_4 was added to neutralize the solution. This solution was diluted to 100 cc. and autoclaved for 10 minutes at 15 pounds pressure. The prepared solution was kept at 6° C., and 1 cc. per liter per day was added to the usual synthetic pond water for one set of assay fishes.

The solution gave a strong protein test and had the odor of fish (amines). As an added control, 10 cc. of N/2 NaOH plus 10 cc. of N/2 H_2SO_4 was diluted to 100 cc. with distilled water, autoclaved with the protein solution, and kept in the icebox. Again 1 cc. per liter per day of this salt control was added to the medium of the lot of isolated fishes that made up the so-called"salt-control."

A material with protein properties was isolated in very small amounts from the usual fish-conditioned water. The protein extract and this material from fish-conditioned water showed the reactions summarized in Table IV.

The protein solution so prepared was used in Experiment O-1. In Experiment O-2, preparation was similar except that 66 fishes averaging 65 mm. in length were extracted. The final solution, after neutralization, was diluted to 180 cc., autoclaved, and stored as before. For Experiment O-4, 20 large goldfishes, 80 mm. long, were extracted. After the first filtration the precipitate was redissolved, re-washed, and reprecipitated two different times, made up to 150 cc., and autoclaved. 0.5 cc. was added per liter, and a 2-liter assay volume was used.

In Experiment O-5, 166 fishes 66 mm. long were extracted as before, except that the second precipitate was thoroughly washed in distilled water, then in alcohol, followed by ether; then desiccated *in vacuo* for 5 days. Of this protein, 1.040 gm. were dissolved in 25.0 cc. of N/10 NaOH plus 175 cc. of distilled water, making a solution of approximately 0.5 per cent, 1 cc. of which was added to 2 liters of water to make a dilution of 1:400,000. The same method was fol-

lowed in later experiments; only the dilutions were varied. Other modified controls were also applied to provide adequate chemical controls.

The summary of results obtained in growth assays is given in Table V. Section A of this table contrasts growth in water plus the protein extract with growth in the usual control; B presents the contrast between the dilute protein extract and the various salt controls; C shows the one test made to date on the relative efficiency of two dilutions of the protein extract; and D compares growth in the

TABLE IV

COMPARABLE TESTS ON PROTEIN FROM AN EXTRACT FROM THE SURFACE OF FISHES AND FROM ORDINARY FISH-CONDITIONED WATER

Tests	Protein Extract	Conditioned Water
Xanthoproteic	Positive	Positive
Biuret	Positive	Positive
Molisch	Positive	Not tested
Millon	Positive	Positive
Hopkins-Cole	Faint positive	Not tested
Lead acetate	Negative	Negative
Glyoxalic	Not tested	Positive
Color, solution	Pale brown	.....
Color, solid	White	.....
Remarks	On drying in air, obtained brown odorous resin-like material difficult to dissolve even in strong alkali with heat	Not coagulated by heat; insoluble in acetic acid, with heating in strong HCl; dissolved in alkaline solution

diluted protein extract with that in the usual strength of conditioned water.

In the experiments available to date there is the following pertinent evidence: (*a*) The difference in growth between assay fishes in water plus protein extract and growth in untreated but otherwise similar control water is 1.41 units, with $P=0.0106$. (*b*) The difference between the growth of fishes in the dilute protein extract and the growth of fishes in all salt controls is 2.61 units, with $P=<0.0006$. (*c*) In the one experiment available there is no significant difference between the growth in 1:400,000 and 1:800,000 parts of protein in the assay water. (If other results should turn out to be

TABLE V

EFFECT OF PROTEIN EXTRACT FROM SKIN OF GOLDFISHES ON GROWTH OF GOLDFISHES

Experiment No.	Days Treated	Extract Conditioned		Control Otherwise Unconditioned		P	Strength of Extract	Salts
		No. of Fishes	Growth	Growth	No. of Fishes			
	A. Protein Extract versus Ordinary Control							
O-3	10	3	1.87	0.33	3	0.068	?	
O-4	20	6	2.16	0.43	9	.....	?	
O-6	20	8	0.62	0.05	8	0.0442	1:800000	
O-7	30	9	1.26	−0.10	10	.....	1:400000	
O-7	30	10	0.76	−0.10	10	.....	1:800000	
O-8	30	10	3.03	−0.05	9	0.0004	1:400000	
O-9	30	10	3.93	3.23	10	0.30	1:700000	
Totals or means		56	1.95	0.54	59	$P=0.0106$		
	B. Protein Extract versus Salt Control							
O-1	20	3	7.1	0.13	3	0.0074		
O-2	20	5	3.68	0.64	5	0.0028		
O-4	20	6	2.16	0.10	6	0.0030		
O-5	20	8	3.8	0.9	8	0.0036	.....	NaCl
				0.7	8	0.0016	1:400000	Amines
				0.4	8	0.0003	1:400000	$CaCl_2$
				0.6	8	0.0003	1:400000	$NaNO_2+(NH_4)_2SO_4$
O-7	30	9	1.26	−0.05	10	0.0040	1:400000	$CaCl_2$
				−0.02	10	0.0040	1:400000	NO_2+NH_4
.....	30	10	0.76	−0.05	10	0.0012	1:800000	$CaCl_2$
				−0.02	10	0.0108	1:800000	NO_2+NH_4
O-8	30	10	3.03	0.25	9	0.006	1:400000	$NaCl_2$
				1.15	9	0.0316	1:400000	$NaNO_2+(NH_4)_2SO_4$
				0.40	8	0.0052	1:400000	Na_2SO_4
O-9	30	10	3.93	4.09	10	Not sign.	1:700000	Salt control
Totals or means	200	61	3.22	0.61	122	0.0006		
	C. 1:400000 versus 1:800000 Protein Extract							
O-7	30	9	1.26	0.76	10	0.27	.....	

TABLE V—*Continued*

Experiment No.	Days Treated	Extract Conditioned		Control Otherwise Unconditioned		*P*	Strength of Extract	Salts
		No. of Fishes	Growth	Growth	No. of Fishes			
	D. Protein Extract versus Conditioned Water							
O-3	10	3	1.87	1.3	3	0.046	?	
O-4	20	6	2.16	1.08	8	None	?	
O-6	20	8	0.62	0.66	7	None	1:800000	
O-8	30	9	3.03	3.23	10	None	1:400000	
Totals or means		26	1.92	1.55	28	$P=0.26$		

consistently in the same direction, the observed difference of 0.5 units more growth in the stronger solution would become significant; this, however, cannot be assumed until tested.) Finally, (*d*) the observed difference in growth in the protein-conditioned water and that in ordinary fish-conditioned water is not significant.

With regard to the food value of the protein-conditioned water we have little evidence. Two tests of the required oxygen were made in Experiment O-10 which showed an oxygen requirement of 1.36 mg. per liter, which is to be compared with a mean oxygen requirement of 0.91 mg. per liter for four accompanying tests in ordinary conditioned water. The strength of the protein extract tested was ten times the assay strength; hence the comparable values with required oxygen, as shown in Table II, would be 0.136, which puts the organic value in the general range of that given in the water conditioned by fasting fishes and then filtered.

If we attempt to explain the stimulation produced by these protein extracts as a result of added food value, we have two major difficulties. The first has already been mentioned in part and concerned very slight growth-promoting power of filtered conditioned water even from fed fishes with approximately four times the required oxygen value of this water. If we assume that the filtered conditioned

water has little power to promote growth because the particles remaining after filtering are too small to be of much food value to the fishes, we have our second major difficulty, since this growth-stimulating protein is probably present as a true solution.

Further, if this is an effect produced by the caloric value of the protein as food, it is extremely potent material, since a dilution of 1:400,000, or even of 1:800,000, has a stimulating effect. It seems more reasonable to assume that the protein acts as some other sort of growth stimulant. We are prepared for the latter assumption by the work of Peebles (1929) on the effect of the protein fraction from a self-conditioned water in stimulating the growth of echinoderm embryos.

This is not the place to give in detail the studies to date on the nature of the protein. In summary these tests show:

Amide N_2 makes 1.26 per cent of the sample.
Melanoidin N_2=0.90 per cent of the sample.
Diamino N_2=1.97 per cent of the sample.
Monoamino N_2=10.43 per cent of the sample.
Total N_2=14.57 per cent of the sample.
Alpha amino N_2=80.55 per cent of the total nitrogen.

One other exploratory test has been made: Ovomucin was prepared according to directions in use in routine work in physiological chemistry at this university. In 17 individual pairs of test fishes there was no significant difference in growth in water plus ovomucin and growth in water plus protein extract; neither was there a significant difference in the same number of cases between fishes in ovomucin and in ordinary control water. The simultaneously made comparisons between fish growth in the protein extract and in untreated control water were statistically significant.

Charcoal treatment.—Preliminary investigation of the effect of treating concentrated conditioned water with charcoal and then testing the growth-promoting power of the treated water and of water conditioned by adding material or materials presumably protein in nature, eluded from the charcoal, have shown that this is a promising lead. The data at hand must not be taken too seriously and hence are not given in detail; they do indicate that the charcoal adsorbs something from the fish-conditioned water which can be

PLATE I

Absorption Spectra

Fig. 1.—*A*, water conditioned by fishes that had been fasting for 5 days; *B*, untreated control water.

Fig. 2.—*A*, concentrated conditioned water from fishes fed liver; *B*, aqueous extract of liver; *C*, protein extract from surface of goldfishes, purified and dissolved in control water.

Fig. 3.—*A*, concentrated conditioned water from fed fishes; *B*, protein extract from surface of goldfishes, purified and dissolved in control water; *C*, water as in *A* after treatment with activated charcoal and filtering.

Fig. 4.—*A*, concentrated conditioned water from fishes fed rolled oats; *B*, aqueous extract of rolled oats; *C*, untreated control water.

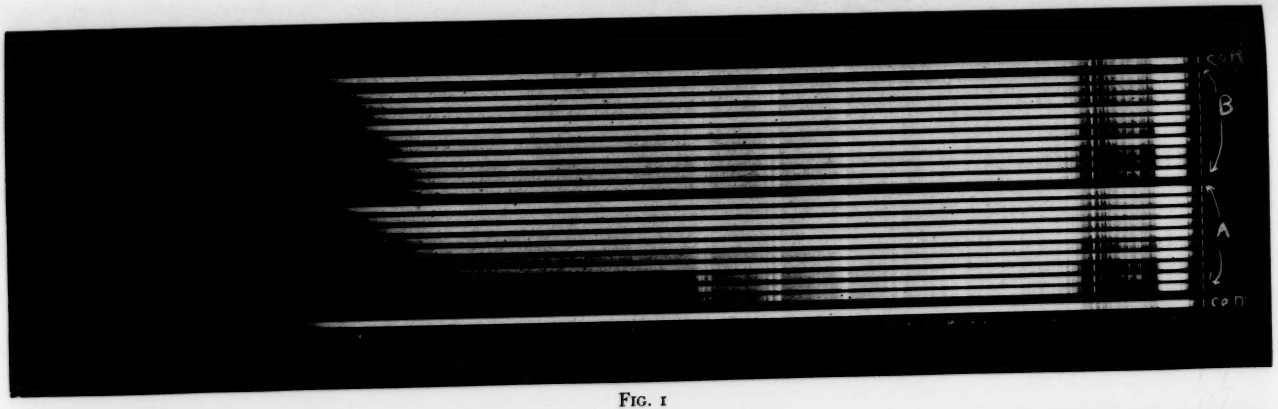

FIG. 1

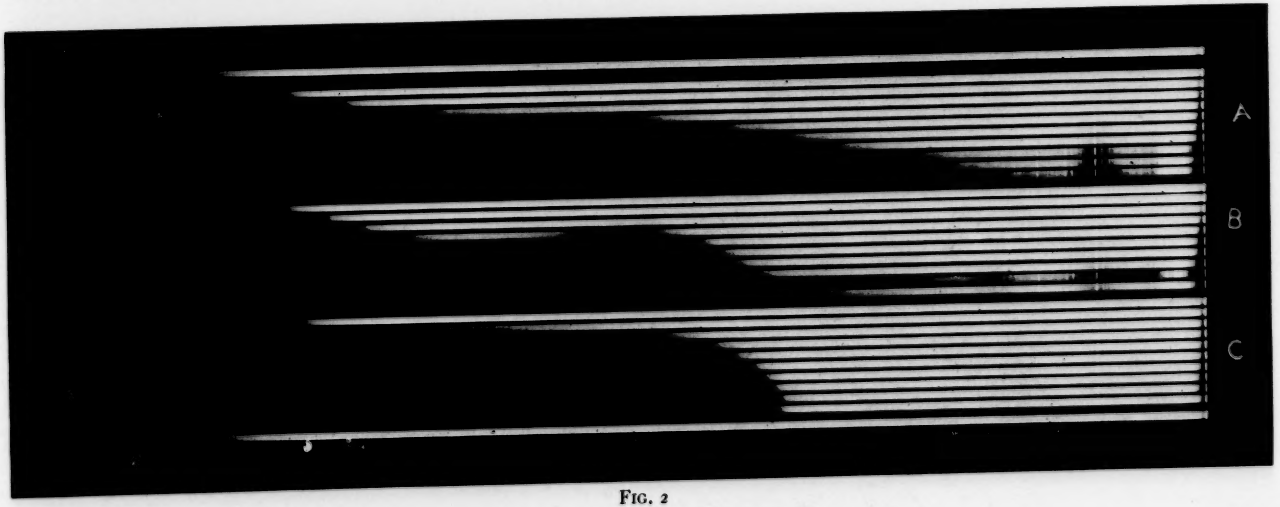

FIG. 2

PLATE I—(*Continued*)

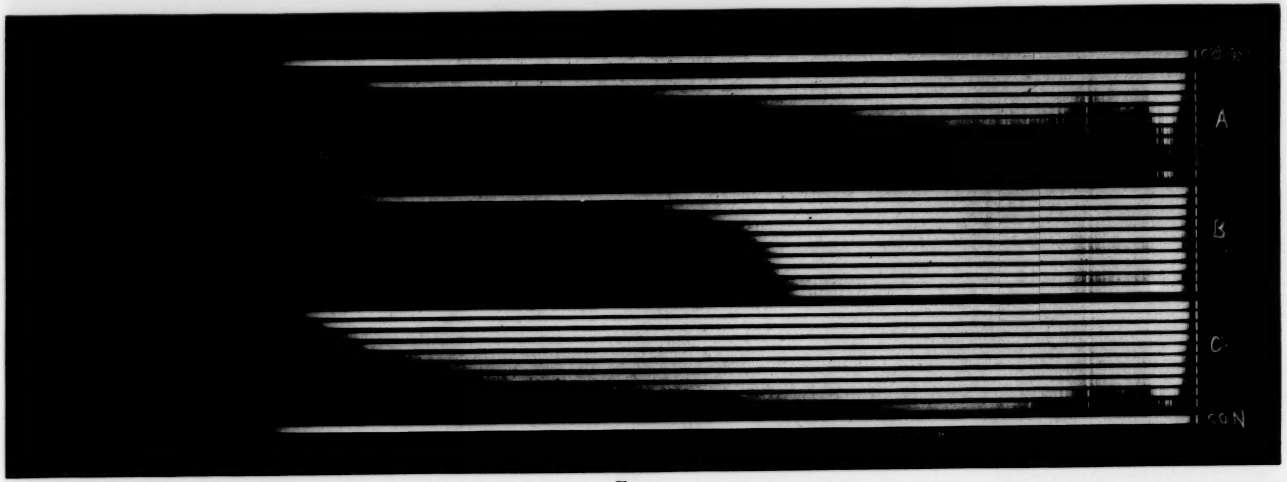

FIG. 3

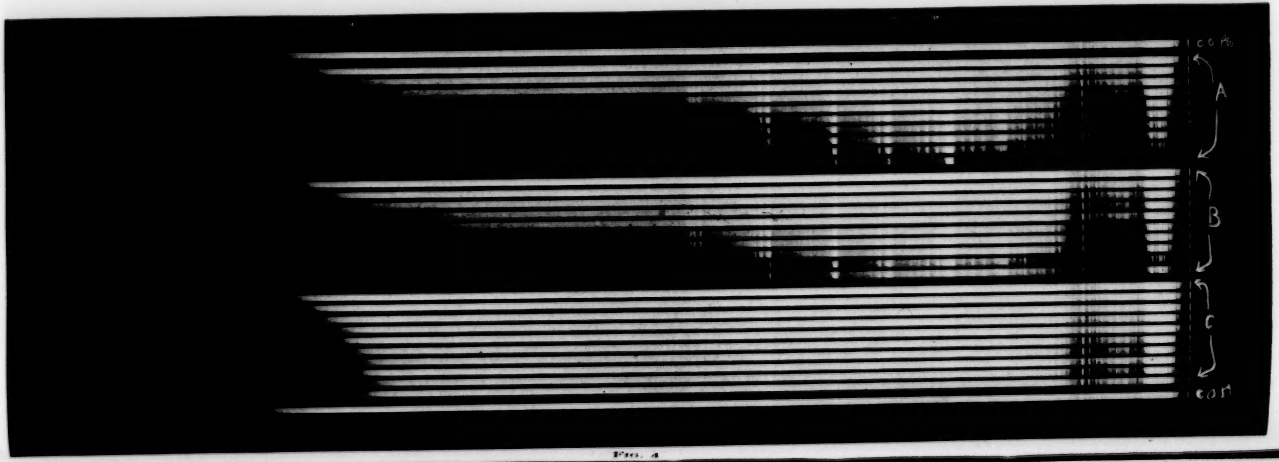

FIG. 4

eluded and will then cause an increase in growth of assay fishes. Pending further investigation, we can only note that this observation again throws doubt on the possibility that the increase in growth in homotypically conditioned water is altogether a result of the addition of food. This attack deserves and will receive further experimental attention.

Spectrographic analyses.—It was considered that a comparison of the absorption spectra of water conditioned by fishes fed on various diets used in relation to the absorption spectra of aqueous extracts of these same diets would test for the presence of food materials in the fish-conditioned water. Again these data are not conclusive by themselves but aid in the interpretation of the biological assays and of the oxygen-absorption or required-oxygen studies.

When conditioning fishes are starved for 5 days or more, the water they have conditioned gives absorption spectrum *A* of Figure 1, Plate I, while that of unconditioned control water gives spectrum *B* of the same figure. When conditioning fishes have been fed on a liver diet for 5 days or more, water they have conditioned has the absorption spectrum *A* of Figure 2, Plate I, while an aqueous extract of liver has the absorption spectrum *B* of this figure, and a protein extract of mucin from the surface of fishes shows spectrum *C* of this same figure. Spectrum *A* of Figure 2 could be a combination of the other two spectra shown in the same figure, and definitely shows evidence of relationship to spectrum *B* of the aqueous extracts of liver.

If we examine the absorption spectra of water conditioned by fishes fed liver (*A* of Fig. 3, Pl. I), the same water treated with activated charcoal (*C* of same figure), and again the absorption spectrum of mucin from the surface of fishes (*B* of same figure), we note that charcoal treatment removes some, but not all, of the material of the same nature as that shown by an aqueous liver extract. Biological assays suggest that the charcoal treatment reduces the growth-promoting properties of conditioned water.

If conditioned water from oat-fed fishes (*A* of Fig. 4, Pl. I) is compared with an aqueous extract of rolled oats (*B* of the same figure), we again find a similarity between the two absorption spectra. By comparing back to Figure 2, Plate I, we are convinced that the

spectra are different from those of liver-fed fishes, from water extracts of liver, or from protein fractions of mucin from the surfaces of fishes. Oat-fed-fish-conditioned water and aqueous extracts of oats are very much alike.

Comparisons of these spectra with the oxygen absorption of the various solutions or suspensions gives a semiquantitative basis which aids in comparisons. These values are shown in Table VI.

TABLE VI

OXYGEN ABSORPTIONS OF WATERS UPON WHICH ABSORPTION SPECTRA HAVE BEEN REPORTED

The Required Oxygen Values Given Here Are Ten Times Those in Table II, Which Were Shown at Assay Strengths

Figure No. of Plate I	Spectrum Letter	Oxygen Absorbed per Liter (in Mg.)
1	*A*	2.90
	B	0.00
2	*A*	11.37
	B	7.39
	C	19.38
3	*A*	11.05
	B	19.38
	C	2.15
4	*A*	5.11
	B	4.05
	C	0.00

These data demonstrate the presence of organic matter of some type in the water conditioned by fed fishes and show the relative lack of such materials in the water conditioned by fasting fishes (*A* of Fig. 1, Pl. I). The evidence regarding the presence of a significant amount of mucin from the surfaces of the fishes in the usual fish-conditioned water is inconclusive; further work at this point is needed and is in progress at this time. These absorption spectra have been confirmed a sufficient number of times to assure us that they are repeatable and are valuable as aids in interpreting our biological assays.

DISCUSSION AND SUMMARY

The present report centers about the question taken as the title: Is food the effective growth-promoting factor in homotypically conditioned water? The problem is being attacked in this laboratory along three main lines, two of which will be reported elsewhere and by others. The most direct approach to the problem is furnished by suspending various amounts of different food substances in water as watery suspensions or extracts and then removing different fractions and testing the different effects on growth of such food-conditioned waters. A second approach is directed toward determining whether a food accessory is introduced as a part of the conditioning process.

The evidence we have presented demonstrates the presence of particles of food in the conditioned water whether whole or screened through openings of about 40 μ in diameter. The nature of the foodstuffs varies with different diets and comes from regurgitated particles and from the faeces. When rolled oats is the chief constituent of the diet, the concentrated conditioned water becomes cloudy with regurgitated oat particles. If the diet is chiefly of liver, the water is more yellowish from minute liver fragments. In all cases, it will be remembered, the conditioning fishes are fed in special containers and are carefully washed after feeding, so that in our experiments no food is placed directly in the food-conditioned water; it comes only from regurgitation or from faeces. When the concentrated conditioned water is diluted down to the strength used in the growth assays, the opalescence is largely lost.

The series of spectrographic analyses of the concentrated fish-conditioned water showed pictures similar to those obtained by direct aqueous extracts from food. There are, in addition, indications of protein effect presumably caused by materials excreted from the surfaces of the conditioning fishes; this effect, however, is masked, so far as spectrographic analyses are concerned, by the food materials introduced by the fishes by regurgitation or from their faeces.

When the whole conditioned water was used as the growth-assay medium, we have direct evidence that the fishes may eat the strings of faeces. Usually the water was sieved so that all particles larger than about 40 μ were fragmented or removed. We know that gold-

fishes of the size used will take particles of charcoal, which had passed through one of the standard sieves, into their alimentary tract; presumably they will take food particles as well.

In addition to gross and microscopic inspection and to spectrographic analyses, the presence of organic matter in the conditioned water is demonstrated by appropriate tests for "required oxygen"; that is, of the oxygen required to oxidize, under standard conditions, the organic material present. The growth-promoting power of the water showed a slight, but significantly positive, correlation with the amount of organic matter so revealed. For the experiments at hand, $r = 0.066 \pm$ a standard error of 0.021. Values of over twice the standard error are usually considered to be statistically significant.

Such a positive correlation between the amount of required oxygen and growth does not necessarily prove that it is the food value of the conditioned water that is effective in promoting growth. The growth promotion may be the result of the presence of some other sort of growth stimulant which is not yet analyzed.

Better evidence that the added food is an important conditioning factor is found in the results obtained by using fasting conditioning fishes as compared either with accompanying controls or with fishes growing in water conditioned by fed fishes. When all the waters are similarly sieved, the assay fishes grow significantly more in the water conditioned by fishes that are fed daily than in water conditioned by fasting fishes. The increase in growth is not as great as would be expected on the basis of the increase in organic matter in the water. They also grow more in water conditioned by fasting fishes than in the untreated control waters.

The importance of food has been checked in another way. The conditioned water both from starved and from fed fishes has been filtered through one or two layers of close-grained filter papers. In the thirteen experiments on filtered "starved" and filtered "fed" conditioned water, growth was greater than in the controls, but not significantly so. In experiments that were made simultaneously, growth did not differ in the two kinds of waters, although the organic matter was almost four times as great in the water from the fed fishes. When all twenty-six of these experiments with filtered conditioned water are compared with their respective controls, there

is a greater growth of 0.36 units in the filtered water, with $P = 0.0027$ and hence significant statistically. This shows either that particles small enough to pass these filters are used as food, and so have significant growth-promoting powers even when present in small amounts, or that there is some other growth stimulant present over and above that possibly furnished by the food value of the filtered conditioned water.

The latter suggestion is further supported by the work with protein extracts from the surfaces of the goldfishes. These have definite growth-promoting powers in *solutions* diluted 1 to 400,000 or even 1 to 800,000. The required oxygen tests show that the organic matter present is approximately equal to that in the filtered conditioned water from fasting fishes. The growth-promoting power is, however, much greater than in such waters.

Further, preliminary experiments on treating fish-conditioned water with charcoal indicate that something is taken from the water by the charcoal which, when eluded, has growth-promoting power. It is not well to dwell too long on these charcoal experiments until more data are available; there are indications, however, that the growth-promoting power of the conditioned water so treated with charcoal is retained, though perhaps lessened. These findings are in accord with absorption spectra from such waters.

The whole amount of evidence demonstrates that a part of the effect of water conditioned by the presence of fed fishes on growth is to be accounted for by its supplemental food value. Food accessories aside, there are enough solid food particles present and apparently available to allow feeding to proceed much beyond the 2 hours allotted to the normal daily contact with supplied food. This is in keeping with the reduced effect of the conditioned water which has been observed when all assay fishes were fed a particularly favorable diet. The experiments do, however, suggest strongly that there is something more than a caloric food factor acting, the biochemical nature and the physiological effects of which await analysis.

With regard to the relative importance of these two aspects of growth promotion by homotypically conditioned water, it is too early to come to a definite conclusion. Perhaps we are unduly rash in even hazarding a guess. On the basis of the relation between the

amount of growth in water conditioned by fed fishes and that in water from fasting ones, and also by making a similar comparison of the growth of the former with that shown in filtered conditioned water, it would appear that under the conditions of our experiments food is some five or six times as effective as is any other growth-promoting factor which is possibly acting. Further experiments may even increase this estimate of the relative importance of the food value of the ordinary fish-conditioned water such as we have been using. When none of the particles have been removed by screening, the mean growth over that shown by accompanying controls is even greater. This is according to expectations if the amount of foodstuffs added by the conditioning fishes is, in fact, the principal growth-promoting factor in such waters.

With hypotonic waters (Allee and associates, 1934) and with certain sorts of toxic materials (Carpenter, 1927; Allee and Bowen, 1932), the conditioning fishes produce changes of other sorts which also make homotypically conditioned water a more favorable medium for fish growth than the untreated water would have been. These studies, together with those reported in the present paper, go far toward placing another phase of mass physiology on a sound experimental basis.

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SELECTION OF FOOD IN PARAMECIUM TRICHIUM[1]

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THE ability of a ciliated protozoon to select its food is still an open question. Schaeffer (1910) described selection in *Stentor*. Day and Bentley (1911) state that *Paramecium* has a limited ability to learn, although these authors were not dealing with food. Metalnikow (1912) concludes that *Paramecium* can learn to select nutritious from innutritious particles by the induction of a condition analogous to a conditioned reflex. Hetherington (1934) states that *Colpidium* sometimes failed to ingest certain types of particles present in the medium which he used. On the other hand, Wladimirsky (according to Calkins, 1933) concludes that Protozoa cannot select nor learn to select food. Calkins (1933) admits selection in such discontinuously feeding ciliates as *Actinobolina* (=*Actinobolus*) but is skeptical of the ability of continuously feeding forms, such as *Paramecium*, to select food.

Among amoeboid forms the situation is clearer. Schaeffer (1916) concludes that *Amoeba proteus* ingests more flagellates, whereas *A. dubia* takes in a greater number of diatoms and desmids. He also (1917) believes *Amoeba* capable "of exercising very nice discrimination in feeding between two particles of different composition—one digestible, the other not. . . . ." Kepner and Whitlock (1920, p. 401) say, "The Ameba seems to have a marked preference for Chilomonas paramecium." Similarly, Mast and Hahnert (1935) conclude that *Amoeba proteus* feeds largely upon *Chilomonas* and *Colpidium*. However, these authors found that under certain circumstances this animal ingests almost any organism which is of appropriate size and is not too active, "but it does not ingest them indiscriminately, e.g., it rarely takes *Monas* if *Chilomonas* or *Colpidium* is available" (p. 270).

From a review of the literature it is apparent that there is practi-

[1] Thanks are due to Dr. B. R. Lutz, of Boston University, for furnishing space and facilities for the work upon which this paper is based.

cally unanimous agreement among workers with amoebae that these forms can and do select their food. On the other hand, no such agreement exists among workers with the ciliates, the greatest doubt centering upon the reactions of the continuously feeding forms, such as *Paramecium*. The following questions, therefore, need to be answered: Can any of the continuously feeding ciliates select their food? If so, do individuals within a species vary in this ability? Is there a similar interspecific variation within a genus? If we admit selection in any form, precisely in what sense do we use the term "selection"?

The present paper submits evidence bearing upon these questions. The principal organism used for experiment was *Paramecium trichium* Stokes, although *P. caudatum* was also used to a limited extent for comparative purposes. *Paramecium trichium* was chosen for study partly because it happened to be available but also because a search of the literature failed to reveal any work which had been done on the nutrition of this form and it was thought that interesting results might be obtained with a species closely related to *P. caudatum*, upon which much work has been done. Variation in the method of ingestion in these two species has already been found (Bragg, 1935*b*), and this fact suggests that variation in selective ability might also be shown.

Some of the confusion apparent from a review of the literature on this subject is probably due to the unqualified use of terms teleological in their implication, such as "choose" and "select." I think no one would attribute conscious selection of food to the Protozoa. The real question is: Do Protozoa ingest certain particles from the surrounding medium to the total or partial exclusion of others of comparable size and abundance but of a different nature? It is convenient to use the term "selection" for this type of phenomenon, and it is in this sense that the term is used in the present paper.

MATERIALS AND METHODS

The animals used in these experiments were collected in Andover, Massachusetts, and were cultured in standard hay infusion at room temperatures. Both *Paramecium trichium* and *P. caudatum* were used, the latter species mostly for comparative purposes.

The method of observation was as follows: A small drop of the culture fluid containing the organisms was placed on a slide, a small amount of the substance to be fed was mixed with this, and a cover slip was sealed over the drop with vaseline. It is to be noted that at all times the animals had free access to their normal food supply, the bacteria in the culture. Observations were made as the animals became quiet about masses of zoöglea or moved slowly along the bottom. The rate of individual vacuole formation was taken with a stop watch and is, in each case, the time elapsing between the instant a given vacuole dropped from the pharynx and the instant the next one dropped.

Substances fed the animals were carmine, carmine mixed with neutral red, and haemoglobin powder. The carmine was that of Colman and Bell. The haemoglobin was that of Eimer and Amend. The neutral red was made up as recommended by Shapiro (1927), 0.5 mg. per 1 c.c. distilled water; but since, when used, the stain was mixed with the culture fluid, the exact strength of it reaching the animals is unknown.

OBSERVATIONS

Paramecium caudatum.—Individuals of *P. caudatum* were found to vary greatly in the number of carmine-containing vacuoles which they would form in a given time. For example, after less than 5 minutes in one preparation one individual held twenty vacuoles containing carmine. Others beside it had one, two, three, or a few more. Carmine grains were continuously being shunted from the buccal grooves of all of the animals as I watched. After 25 minutes in this preparation some individuals had no carmine-containing vacuoles: others had up to thirteen. After 1 hour and 15 minutes, thirty-five animals, taken at random, averaged 14.83 vacuoles containing carmine. Of these, eleven individuals contained less than ten; fourteen contained from ten to nineteen; and ten had more than nineteen. One animal contained twenty-nine, the highest number recorded. All of these animals contained bacterial vacuoles of the usual type as well, many of which must have been formed while the carmine was present. These observations were repeated many times and always with comparable results. Therefore, it is concluded that *P.*

caudatum has a limited selective ability which is differentially distributed among individuals.

Paramecium trichium.—The results with *P. trichium* indicate that this species selects bacteria from carmine even more readily than does *P. caudatum*. When individuals are taken at random and the number of carmine-containing vacuoles is counted in each after various lengths of time, some individuals are seen to have no carmine-containing vacuoles. Others contain but a few of them. A few individuals have large numbers. Table I gives the observed data in a typical case. Further data are not given since they were all consistent with this.

TABLE I

NUMBER OF CARMINE-CONTAINING VACUOLES IN INDIVIDUALS OF *Paramecium trichium* AFTER VARIOUS LENGTHS OF TIME IN A SINGLE PREPARATION

Time (Minutes)	Number of Vacuoles	Average
2–12	3, 5, 2, 2, 1, 0, 0, 3, 2, 3, 3, 2, 0, 4, 3, 3, 3, 3, 3	2.37−
20–30	0, 1, 1, 3, 2, 3, 4, 1, 7, 5, 3, 3, 2, 6, 4, 2, 3, 2, 4, 3, 2, 2	2.86+
30	10, 16, 0, 6, 10, 21, 9, 22, 11, 25, 12, 18	13.33+
35–45	3, 0, 3, 3, 2, 1, 1, 2, 2, 3, 1, 1, 1, 1, 4	1.87−
75	1, 4, 4, 2, 3, 5, 6, 4, 0, 13, 1, 4, 3, 22, 0, 6, 2, 11, 2, 13, 1, 4, 10, 1, 11, 2, 6, 1, 15, 9, 2, 6, 5, 1	5.29+

These results may be interpreted in two different ways: either the animals, with some exceptions, took in very little carmine, or they threw it out about as fast as they took it in. The latter possibility has to be kept in mind, for I have seen a carmine-containing vacuole egested by *P. trichium* within 15 minutes of its formation. Furthermore, Metalnikow (1912) noted that in *P. caudatum* food vacuoles containing innutritious particles were retained for relatively short times in the body of the organism. The fact that there were so few animals which had large amounts of carmine in their bodies and the fact that streams of carmine grains could be seen being thrown out of the buccal grooves of the animals as they fed lead me to favor the first interpretation. In this connection it is also to be noted that all of these animals contained bacterial vacuoles having no carmine in them.

Greenwood (1886–87) studying *Amoeba*, suggested that when this

organism ingests carmine it does so incidently, the stimulus being supplied by a food organism in the immediate neighborhood. To test this matter in *Paramecium trichium*, individuals in a single preparation were taken and their carmine-containing vacuoles recorded in two sets, (1) those containing large masses of carmine as opposed to (2) those having no more than four grains. Table II shows the result in a typical case. Nearly 75 per cent of the vacuoles containing carmine held but a few grains each, and most of these were very small grains which could easily have been swept into the pharynx under the stimulus afforded by the bacteria without being perceived by the animal.

TABLE II

NUMBER OF VACUOLES CONTAINING LARGE AMOUNTS OF CARMINE AS COMPARED WITH THOSE CONTAINING NO MORE THAN FOUR GRAINS EACH IN TEN INDIVIDUALS OF *Paramecium trichium* AFTER ½ HOUR IN A SINGLE PREPARATION

	Individuals										Total	Percentage
	A	B	C	D	E	F	G	H	I	J		
Total vacuoles	9	14	7	23	8	24	18	8	30	20	161	1.00
Large amounts	6	7	0	7	0	5	6	2	6	4	43	0.267+
Small amounts	3	7	7	16	8	19	12	6	24	16	118	0.732+

A further test was made by tabulating all of the food vacuoles within the animals, recording the number of vacuoles containing no carmine, those containing small amounts, as above, and those containing large masses. A typical case is shown in Table III. Of the ninety-three vacuoles reported in the table, approximately 48 per cent had small amounts of carmine, and only about 12 per cent contained large masses.

When neutral red and carmine were presented to the animals together, a most surprising result was obtained. Every vacuole now formed had large masses of carmine, and carmine grains could clearly be seen pouring down the buccal grooves of the animals and entering the vacuoles. Furthermore, relatively little carmine could be seen to be swept out of the buccal grooves of the feeding animals. I at first thought that perhaps the red color of the vacuoles due to the

dye was being confounded with the red color of the carmine grains. Long and careful observations, however, convinced me that this was not the case. The angular nature of the carmine grains could clearly be seen within the vacuoles.

TABLE III

NUMBER OF VACUOLES OF *Paramecium trichium* CONTAINING LARGE AMOUNTS OF CARMINE AS COMPARED WITH THOSE CONTAINING SMALL AMOUNTS OR NO CARMINE AFTER ½ HOUR IN A SINGLE PREPARATION

	Individuals					Total No. Vacuoles	Percentage
	L	M	N	O	P		
Total vacuoles	24	22	11	20	16	93	1.00
Large amounts	3	6	2	0	0	11	0.118+
Small amounts	15	6	4	4	16	45	0.483+
No carmine	6	10	5	16	0	37	0.397+

The food vacuoles so formed were of larger size than usual, as judged by the eye. Also, the rate of their formation immediately decreased about 50 per cent. After from 12 to 48 hours in the preparation, the rate had again increased, until now it was greater than the initial rate. The figures of a typical case are given in Table IV.

TABLE IV

RATE OF FOOD-VACUOLE FORMATION IN *Paramecium trichium* AFTER VARIOUS LENGTHS OF TIME IN A NEUTRAL-RED-CARMINE PREPARATION AS COMPARED WITH THE RATE WHEN THE ANIMALS INGEST ONLY MATERIALS FROM THE CULTURE

Conditions	No. of Vacuoles	Time (Seconds)	
		Range	Average
Fed culture only	30	11.8–70.0	25.1
Carmine and neutral red, 10–60 minutes	36	14.0–99.0	50.6
Carmine and neutral red, 12–48 hours	36	8.4–23.2	16.9

My records also show that neutral red had a steadying effect upon the time required for the formation of individual vacuoles; that is, the time taken by each vacuole to form tended to approach more

nearly to the average time taken for all vacuoles under the condition studied.

Limited data, given in Table V, do not show that neutral red has the same effect upon *Paramecium caudatum*. It should be noted, however, that this organism was not subjected to carmine and neutral red together.

TABLE V

RATES OF FOOD-VACUOLE FORMATION IN *Paramecium caudatum* UNDER VARIOUS CONDITIONS

Conditions	No. of Readings	Average Time (Seconds)
Culture only	56	41.1
Carmine added	35	44.4
Neutral red added	36	44.1

Haemoglobin powder was completely rejected by *P. trichium*. In a typical preparation, for example, the animals were under constant observation for 35 minutes and not a single haemoglobin-containing vacuole was seen. At the same time, many vacuoles containing bacteria were seen to form, and all of the animals were throwing haemoglobin granules from the buccal grooves. Several preparations containing varying quantities of haemoglobin and organisms from different cultures gave the same result.

When the animals were first placed into the haemoglobin preparations, many responded by avoiding reactions of the type described by Jennings (1931) and by eventually congregating in the region of the slide containing the least haemoglobin. After a time they became quiet here and continued to feed upon the bacteria in the culture. These facts suggest that either some small amount of a soluble substance was present as an impurity in the haemoglobin or that the haemoglobin itself was slightly soluble. In any case, the animals failed completely to ingest it.

In addition to the evidence already presented, another type of observation indicates that certain individuals of *Paramecium trichium* may show very marked selective action. On two occasions individuals were seen whose vacuoles contained large bodies some of which so closely resembled the flagellate *Chilomonas paramecium*,

which was abundant in the culture, that I had no hesitation in believing them to be this organism. The first of these animals was found in a haemoglobin preparation. It contained twenty exceptionally large food vacuoles, each containing a single *Chilomonas*. This specimen had no typical food vacuoles. The second case of this sort was seen the following day in an animal from the same culture from which the first had come. In this case nothing had been added to the culture. The animal had several normal food vacuoles containing bacteria and one large vacuole which held a *Chilomonas*. Careful search of all of my cultures failed to reveal other specimens of this type.

As showing selective action on the part of these paramecia, these observations are of some significance. The mouth of *Paramecium* is not large in any species of the genus, and we are here dealing with the smallest *Paramecium* yet described. Its length is given by Kudo (1931) as from 70 to 100 μ, while that of *Chilomonas* is given as from 30 to 50 μ. Assuming that we have the largest *Paramecium* and the smallest *Chilomonas* within these limits, it is still difficult to understand how the one could ingest the other without the mouth becoming considerably stretched; and the stretching of the mouth during ingestion, while quite common in normally carnivorous ciliates, has not been reported, so far as I am aware, in *Paramecium*. These flagellates must have been ingested, therefore, with considerable difficulty. These observations prove conclusively that at least some individuals of *P. trichium* are able to select their food. Such marked ability to select food organisms must be extremely rare, however, since I have watched thousands of food vacuoles form in *P. trichium*, *P. caudatum*, and *P. aurelia* and have never seen any indication of this sort of thing except as noted above.

It would be of extreme interest to observe a *Paramecium* ingesting so large an organism, and to compare the movements of the food vacuole with those normal to the organism, as recently described (Bragg, 1935*a*).

SUMMARY

Paramecium trichium was fed carmine, carmine mixed with neutral red, and haemoglobin powder, by mixing each with the culture fluid containing the organisms. Observations showed that more food

vacuoles containing no carmine, or but a few grains of it, were formed than those holding large masses. Individuals varied considerably in this respect. Neutral red inhibited the selective action. It also had the effect of at first slowing the rate of vacuole formation and markedly increasing the size of individual vacuoles; later the vacuole formation rate was greatly increased. Haemoglobin was totally rejected by the animals. Two individuals were found which had ingested *Chilomonas*, one of them to the total exclusion of other materials.

It is concluded that *Paramecium trichium* has a limited ability to select its food, that individuals vary in the amount of selective action which they show, and that the mechanism through which the selection works involves the direction and probably the rate of beating of the peribuccal cilia.

More limited observations on *P. caudatum* confirm the conclusions of those who have maintained that this species is able to select its food. They also indicate that this species has less of this ability than has *P. trichium*. Incidently, this difference between these two species, when added to the difference already found in their ingestive mechanisms (Bragg, 1935*a*), may be of some importance to those interested in tracing nutritional conditions in other closely related species.

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QUALITATIVE AND QUANTITATIVE CHANGES IN RADIOSENSITIVITY OF GRASSHOPPER EGGS DURING EARLY DEVELOPMENT[1, 2]

(Three figures, one plate)

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THE generalization of Bergonie and Tribondeau (1906), as it has been applied to embryology, indicates that as an organism develops it becomes steadily more resistant to radiation. There have been several recent papers which reveal certain instances when the embryo is more resistant, and certain others when the developing organism is more susceptible than the foregoing generalization would indicate. P. S. Henshaw (1932) and P. S. and C. T. Henshaw (1933) point out that gastrulation in the *Drosophila* egg is a particularly resistant stage to radiation capable of penetrating uniformly to all parts, and that this increased resistance may be related to the paucity of cells in active mitosis at this stage of development. Scott (1934) presents data on eggs of *Calliphora*, and he agrees with the results of the Henshaws (1933). The effects of irradiation appear very slowly, and some recovery is noted if eggs of the grasshopper are treated during the prolonged period of developmental inactivity (Evans, 1934). It appears from the foregoing reports, as well as from many others, that developmental inactivity lowers the susceptibility of organisms to Roentgen rays. However, the later papers of Henshaw and Francis (1933, 1936) indicate that the exact relationship is not clear.

Scott (1934) reports that in *Calliphora* the eggs in the stage just preceding the blastula and also preceding the gastrula are extremely

[1] The writer wishes to express his appreciation to Professors J. H. Bodine, H. D. Kerr, and E. Slifer for their advice and constructive criticism during the course of this investigation.

[2] Aided by a grant from the Committee on Radiations of the National Research Council and by the Rockefeller Foundation Fund for Research on Physiology of the Cell.

susceptible to irradiation. These increases in vulnerability are explained on the basis of the complexity of cell changes which must occur at these stages. Packard (1935) also finds that in *Drosophila* the eggs are particularly susceptible just before blastulation and during invagination, when they are undergoing physiologic and morphologic changes.

The investigation of changes in susceptibility of grasshopper (*Melanoplus differentialis*) eggs to Roentgen rays has been undertaken in the hope of making some contributions to the knowledge of mechanisms involved in ontogenetic development, as well as of learning more concerning the biological effects of Roentgen radiation. The development of the grasshopper egg may be divided into three stages: (1) pre-diapause, (2) diapause, (3) post-diapause. The pre-diapause development involves the formation and early differentiation of the embryo. The diapause is a period of developmental inactivity. During post-diapause development the embryo goes through blastokinesis, engulfs the yolk, and hatches. The reader is referred to the following papers for details concerning the morphological and physiological changes involved: Bodine (1932) and Slifer (1932).

MATERIALS AND METHODS

The radiation was delivered at 130 kv., 5 ma., 30 cm. distance, and was filtered with 2-mm. cardboard. The dosage was applied in one treatment, and the amount was increased by lengthening the exposure time. The intensity was usually 200 roentgens per minute, measured in air.

The eggs were removed from the sand immediately after they had been laid, and were incubated at 25° C. They were irradiated at room temperature (approximately 26° C.) on moist filter paper on a strip of gauze suspended 3 feet above the floor to avoid scatter.

The early development of the grasshopper, *Melanoplus differentialis*, has been studied histologically by Slifer and King (1934). It is possible by the use of the foregoing paper (and from communication with Dr. Slifer) to apply the following chart relating the internal structure of the egg to its age at 25° C.:

Freshly laid eggs—first maturation division

9 hours—both polar bodies given off and from 1 to 3 nuclei present

1 day—from 61 to 32 segmentation nuclei present
2 days—many segmentation nuclei, some in peripheral protoplasm
3 days—definite germ band from one to two cell layers thick
4 days—germ band from two to four cell layers thick
5 days—invagination and formation of germinal layers

The embryo has definite form after 5 days and may even be dissected from the egg for closer observation. From this time on to the twenty-first day the embryo enlarges and differentiates steadily. The reader is referred to Slifer (1932) for the details of the morphological stages.

EXPERIMENTAL

Figure 1 gives the results of a series of experiments in which eggs of different ages at 25° C. are exposed to dosages of 100, 200, 300,

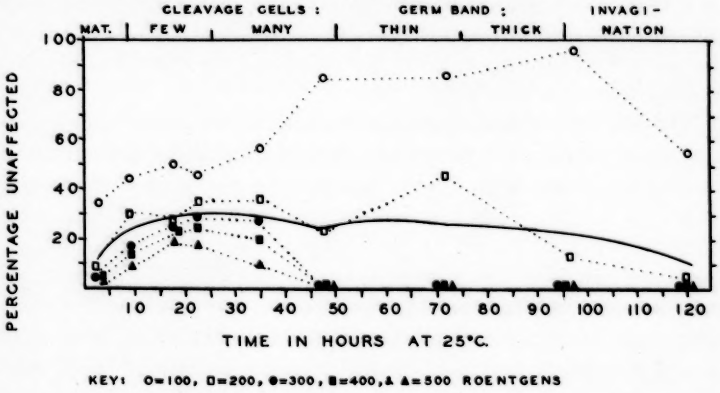


FIG. 1.—Graph showing changes in resistance to Roentgen rays during the first five days (at 25° C.) of development. The ordinates indicate number of eggs containing complete embryos, when examined at the time of diapause, in percentage of the controls. The abscissas show the time in hours. The condition of the egg at the time of irradiation is shown at the top of the graph. The heavy line represents the average resistance to all five of the dosages indicated in the key.

400, and 500 roentgens. Each symbol represents the resistance of eggs of an age to a dosage. The eggs are examined at the time of diapause. The controls at this stage are uniform, and any structural injuries of the irradiated embryos are easily recognized. The number of eggs in each lot varies from 42 to 122, and each symbol represents

the average of several lots. The solid line in the graph represents the average resistance to all five dosages and indicates in a general way the changes in susceptibility during the first five days of development. It may be seen from the graph that the general resistance is very low during the first 3 or 4 hours, but increases after the ninth hour and remains at about the same level until the fourth day. The resistance to 200 roentgens appears to follow about the same curve as the average of all five dosages, being lowest (after ninth hour) during the fifth day. It appears from an examination of the results of irradiation with 100 roentgens that the resistance is lower during the first two days than during the next two. The contrary seems to be the case following heavier irradiations (300, 400, and 500 roentgens). One way of expressing this change is to say that during the first two days a few of the eggs are extremely susceptible and a few are extremely resistant, whereas during the next two days the resistance of all the eggs is about the same and the effective dosage is 300 roentgens, or above.

The above-mentioned graph expresses the variation in only one end-point, namely, the percentage of eggs uninjured when examined at the time of diapause. The variation in the character of the injury, as well as the frequency of its occurrence, is shown in Figure 2. This figure consists of twelve graphs, representing the relation of 100, 200, 300, 400, and 500 roentgens to the extent of injury, and the percentage of its occurrence in eggs from 1 to 13 days of age. The percentage of eggs completely unaffected is indicated by the height of the solid black columns. On the first day the general resistance is not high, but a few eggs are not affected by even 500 roentgens. After the second day of development all eggs are susceptible to more than 300 roentgens, and this condition remains until the eighth day. All eggs are injured by dosages above 400 roentgens up to the tenth day, and all are affected by more than 500 roentgens until the eleventh day. This would seem to indicate that on the first day a few eggs are extremely resistant; and again, beginning with the eighth day, more resistant eggs are found. The height of the solid columns gives the percentage of resistance to the various dosages. It will be seen that on the second, third, and fourth days the resistance to the lower dosages increases markedly and then drops on the

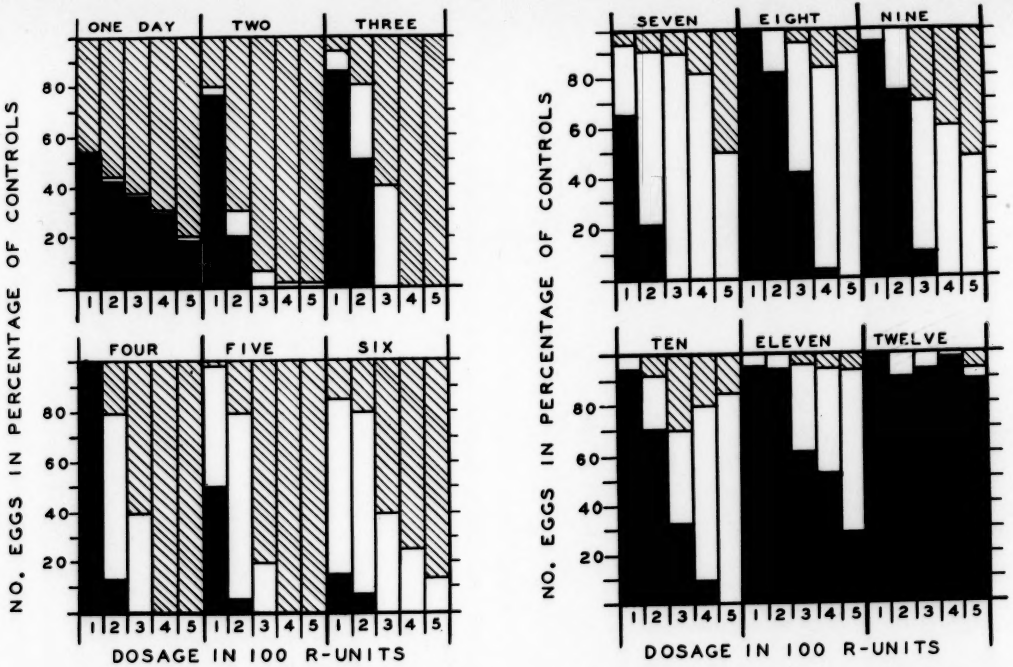


FIG. 2.—Twelve block graphs indicating the results of a series of experiments in which eggs of different ages were irradiated and the extent of injury to individual embryos determined at the time of diapause. Incubation was at 25° C. The solid blocks indicate the number of eggs containing complete embryos, in percentage of controls. The shaded blocks indicate the percentage of eggs which contained no embryos at all. The unshaded areas represent the percentage of eggs in which the embryos were partially injured. The age of the eggs (in days) in each experiment is shown at the top of the graph. The dosages are indicatted at the base, in terms of 100 roentgens.

fifth and sixth days. It appears that on these two days (fifth and sixth) all of the eggs are particularly susceptible to even small dosages. Beginning with the seventh day the resistance to the lower dosages begins to pick up again.

Let us now consider the extent of injury to the individuals at the various stages. The shaded areas indicate the percentage of eggs which contain no embryos at all when examined at the time of diapause. The blank areas represent the percentage of eggs which contained partially injured embryos when examined. It will be seen that on the first and second days the irradiation either destroys the entire embryo or it apparently does not affect it at all. Beginning with the third day, some differentiation has evidently taken place, in that cells are now differentially susceptible. The number of partially injured embryos increases with the dosage, and with ageing more and more of the affected eggs contain embryos only partially destroyed. Eggs which had been irradiated on the third and fourth days, when examined at the time of diapause, contain many embryos with the whole head, thorax, or abdomen missing. After the fourth day, the irradiation results in shrinking and histolysis of parts, but the injury is more localized. As the dosage is increased, the injury is more extensive. In summary, the graphs in Figure 2 show that the effects of roentgen irradiation parallel to a certain extent the progress of differentiation. With the onset of differentiation the parts of the organisms become differentially susceptible. In general, as the embryos become more differentiated, they are more resistant to light dosages; but there is one stage during the fifth and sixth days where the eggs are all very susceptible. This increased susceptibility is apparently related to the histological picture, which shows that at this time the embryo is in the process of invagination and formation of the rudimentary structures of the embryo (Slifer, unpublished). This finding indicates that in the grasshopper the resistance of the embryo does not always increase steadily, and in stages involving extensive differentiation of tissues it is unusually susceptible.

Another indication that this period of increased sensitivity is related to differentiation is the appearance of abnormal structures in embryos irradiated during this period. Figure 3 shows typical morphological aberrations that are found in the irradiated eggs examined

some time after the onset of diapause. (No. 1 is a drawing of a control at the time of diapause.) At the present time the writer is unable to correlate exactly either dosage or time of irradiation with a particular type or degree of abnormality, although certain trends are evident. The production of abnormalities is most frequent during the fifth, sixth, and seventh days. Only 5 eggs out of 1,527 irradiated between the first and fourth days produced abnormalities. One hundred and thirty-four out of 1,176 rayed between the ages of 5 and

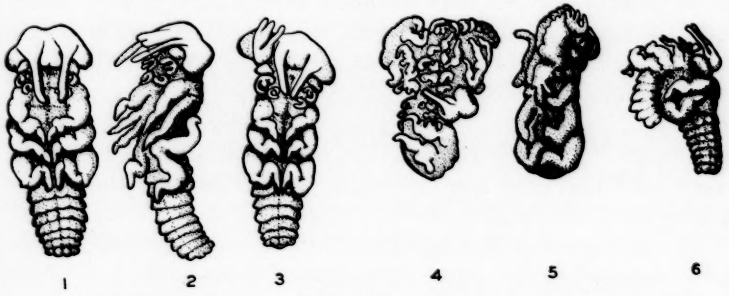


FIG. 3.—Drawings of embryos showing some of the anomalies found in embryos irradiated in the five- to six-day stages. No. 1 is a ventral view of a control at the time of diapause. No. 2 is an irradiated embryo (same view, same scale) showing bifurcation of right mesothoracic leg and of left metathoracic. No. 3, another experimental with small aberrant growth protruding from right side of head. No. 4, disintegration of original abdomen, and enormous secondary growth from head region apparently in three sets of appendage-bearing somites. No. 5, anterior outgrowth from head resembling a secondary embryo. No. 6, complete embryo occupying region of original head and mouth parts.

8 days resulted in anomalies. Out of 1,575 eggs rayed between the ages of 8 and 13 days, not one exhibited anomalies such as indicated above. It therefore appears that the production of the anomalies may be related to invagination and the formation of the basic structures which are in process during the fifth, sixth, and seventh days.

It was noticed that the serosa and cuticle are present in many of the irradiated eggs at diapause even though the embryo has been completely destroyed. It is necessary to expose eggs of 1 day of age to 1,000 roentgens in order to prevent the formation of serosa and cuticle. Even this dosage fails to prevent their formation when 3-day eggs are treated, although the cuticle is not visible until several

days later. This dosage (1,000 roentgens) is twice that necessary to inhibit the formation of the embryo; so these cells that do not go into the formation of the embryo proper must differentiate early and are apparently very resistant.

Several groups of eggs were irradiated on the first and second days of development with the micropyle end of the egg containing segmentation nuclei and surrounding protoplasm) protected with lead. All of these eggs contained complete embryos when examined, at the time of diapause, even following an irradiation of 1,000 roentgens. When the micropyle end of the egg is uncovered and the other half of the egg protected with lead, the results are the same (complete inhibition of embryo formation) as when the whole egg was irradiated. It appears, therefore, that the nuclei are developmental centers and that nothing is contained in the yolk at this time which, when irradiated (700–1,000 roentgens) affects the formation of the pre-diapause embryo.

The procedure of partially protecting the egg was extended to later stages when the embryo is definitely formed. Pin holes were punched in a sheet of lead and the eggs were fastened to the lower surface, permitting certain regions to be exposed to the beam of radiation. A series of 5- and 6-day eggs was irradiated with the following regions exposed: (1) just the micropyle, (2) region just nearer the middle of the long axis of the egg, and (3) center of egg. Typical results are shown in *B* and *C* of Plate I. Some eggs that had been kept at 5° C. long enough to permit immediate post-diapause development at 25° C. were also partially protected during irradiation. The disintegration appears only in regions exposed; but in cases where the region is as extensive as those shown in 1, 2, 3, of *E*, Plate I, the organisms do not go beyond the first few days of post-diapause development. In a few cases the injury was localized to the mouth parts, and such embryos hatch but die within a few days. These experiments serve chiefly to show some possibilities of applying this method to a study of the mechanics of early development.

DISCUSSION

It is difficult to compare the changes in susceptibility of the eggs of the grasshopper with those reported for *Calliphora* (Scott, 1934)

and for *Drosophila* (Henshaw P. S., 1932; P. S. and C. T. Henshaw, 1933; Packard, 1935). The comparison is made unreliable because of the differences in the development of the organisms and the experimental methods employed. It is apparent, however, that all agree that increases in susceptibility are related to (1) cellular activity and (2) complex cellular differentiation.

The finding that the resistance of grasshopper eggs to a dosage of 100 roentgens increases with development during the first four days agrees with the conception of Bergonie and Tribondeau (1906). The fact that 3–4-day eggs are partially affected by dosages of 300–500 roentgens, whereas at least a few of the earlier (1–2-day) eggs are apparently unaffected by these dosages, appears to be inconsistent. There is a possible assumption, however, which will make this apparent exception more in accord with what one would expect. This assumption is that unaffected cells of the 1–2-day stage are able to take the place of the ones destroyed by the irradiation. As differentiation progresses, the number of these totipotent cells naturally decreases.

The increased susceptibility during the fifth, sixth, and seventh days may be explained as a peculiar interplay of cell susceptibility and regenerative forces. The morphological changes at this time indicate that an extensive differentiation of the embryo is in progress. The increased vulnerability to even low dosages may be due to the complexity of cell changes involved, as is suggested by Scott (1934) and by Packard (1935), and may be complicated by changes in the regenerative power of certain cells. The secondary growths of embryos irradiated at this stage indicate the presence of cells possessing the powers of renewed growth even though the proper differentiation may be upset. There seems to be no evidence at the present as to whether the "regeneration" is related to the presence of certain cells which have retained their early embryonic structure and power of further differentiation or whether advanced cells dedifferentiate and reform other types of cells.

The general increase in resistance as the embryos develop may be explained by either of two implications of the law of Bergonie and Tribondeau (1906). The first is the one usually referred to; and it states, in substance, that the resistance of an embryo is (1) propor-

PLATE I

Photomicrographs of grasshopper embryos showing results of partially protecting eggs from irradiation.

A. Controls on the sixth to seventh days (25° C.)

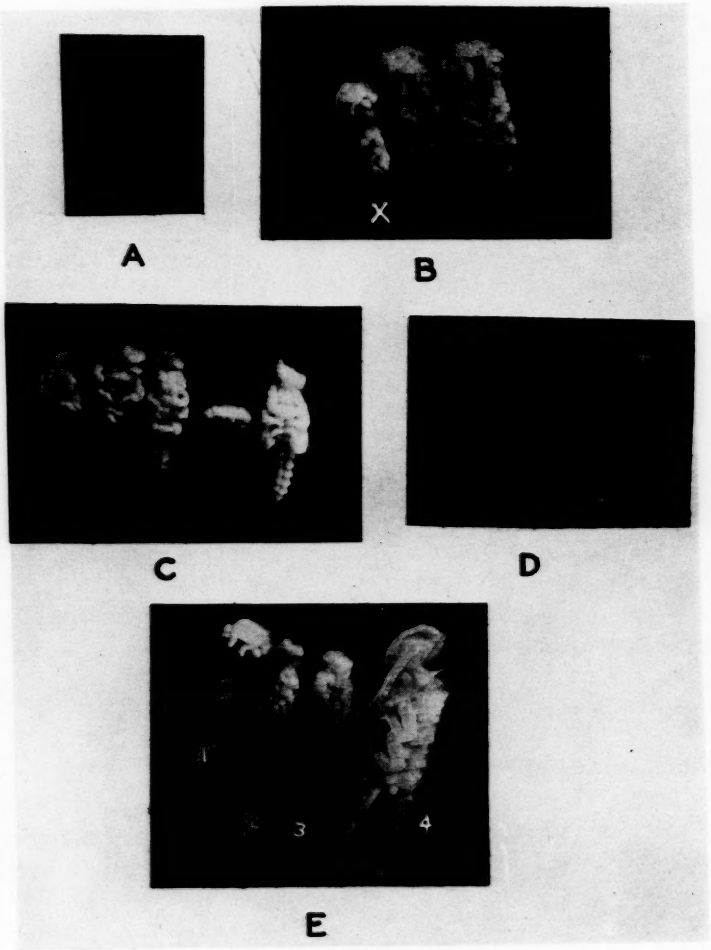
B. Results of irradiating (1,000 roentgens) center of six-day eggs. *X* is irradiated embryo showing abdomen to be missing. Other two embryos are controls at the time of diapause.

C. Embryos from eggs irradiated on the seventh to eighth days with only the micropyle exposed. Note injury to head region.

D. Controls after two months at 5° C. (in seventh to eighth day stages) are now capable of uninterrupted development at 25° C. until hatching.

E. Results of irradiating (1,000 roentgens) eggs similar to those of *D* with organisms partially protected with lead. No. 1 is an embryo taken from an egg irradiated with micropyle region protected. Nos. 3 and 4 are from eggs irradiated with micropyle region exposed. No. 4 is a control at the time these eggs were examined.

PLATE I



tional to its degree of differentiation and (2) inversely proportional to its potentiality for further development. The second viewpoint, which the writer wishes to emphasize, is the "potentiality of further development." It is generally observed that injury may not appear for some time following irradiation, and that it may be made to appear much sooner by increasing the dosage (Evans, 1934). Also, irradiated organisms which do not appear to be injured as long as they are developmentally inactive suddenly exhibit harmful effects on the initiation of development. It therefore becomes apparent that increased resistance of older organisms may be observed because they are nearer the time of the end-points employed and have not had the time or opportunity to express the full extent of the injury.

CONCLUSIONS

The following conclusions are based on the results of experiments in which grasshopper eggs of different ages are irradiated and examined at the time of diapause:

1. During the first two days a few eggs are killed by low dosages, but some are resistant to even 500 roentgens. When the irradiated eggs are examined, they contain either a complete embryo or none at all.

2. The resistance to low dosages increases during the second and third days, but the number of complete embryos found after irradiations of 300–500 roentgens drops markedly. The embryos are not completely missing in the affected eggs. However, the injury is still extensive, and missing parts relate to a whole abdomen, thorax, set of mouth parts, or head.

3. From the third to the fifth day the germ band is thickening and the serosa is formed from the blastoderm. Irradiation at this time affects all of the embryos if it is of more than 300 roentgens. If it is less than 200 roentgens, hardly any of the eggs are affected. The injury at this stage is localized, chiefly to the more rapidly growing parts; but if the dosage is higher, the growth of the whole body is retarded.

4. The embryos are undergoing extensive changes in differentiation between the fifth and seventh days, and this stage is peculiarly sensitive to Roentgen rays. Although nearly all eggs are affected by even light dosages, certain regions possess the ability to develop more growing tissue, although the correct differentiation of this new tissue may be affected.

5. Following these extensive differentiation changes, the embryos become more resistant, and the effect of increasing the irradiation is evidenced by more extensive injury to individuals.

6. The foregoing conclusions, together with some preliminary experiments of another nature, indicate that the law of Bergonie and Tribondeau is not sufficient to explain all changes in susceptibility during development. However, it has implications which are strengthened by the foregoing findings, because the two factors which it recognizes (growth-rate and differentiation) are involved in even the detailed changes noted above.

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OXYGEN CONSUMPTION AND RATES OF DEHYDRATION OF GRASSHOPPER EGGS (ORTHOPTERA)[1]

(Ten figures)

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INVESTIGATIONS regarding the effect of the removal of water from living materials may be classified into three general categories: first, the effect of desiccation on behavior of animals; second, the vital limit of desiccation and the rate of water loss; third, the effect of dehydration on physiological processes, such as respiration, etc.

In the first of these, results have, in general, shown an increased irritability as the first response of animals to desiccation (Shelford, 1913; Weese, 1917; Hamilton, 1917; Chenoweth, 1917; Hayes, 1927; and others).

Hall (1922) and Ludwig (1936) have reviewed the literature concerning the vital limits of desiccation; and from this, one is impressed by the great variability among organisms in their ability to withstand water loss. The rate of water loss is also an important factor. Durig (1901), for example, observed on the common European frog that a vital limit of desiccation was reached at 15 per cent weight loss, if water loss was rapid, while 30–39 per cent of the initial weight could be lost if the drying was slower. Gunn (1933) observed the same for the cockroach (*Blatta orientalis*), 44 per cent weight loss being the limit for slow and 32 per cent for rapid desiccation.

The rate of water loss by the same animal at different stages of development has been studied by relatively few workers. Zavadovskii (1932) found larvae of Trichostronhylidae became increasingly resistant to drying as they became older. Ludwig (1936), using the Japanese beetle (*Popillia japonica*), reports an increasing resistance

[1] Aided by a grant from the Rockefeller Foundation for work on the physiology of the normal cell.

to desiccation in the first, second, and third instar stages, with the pupae being the most resistant.

Both increases (Warburg, 1909; Caldwell, 1925; Hess, 1930; Buchanan, 1931; Duryee, 1932) and decreases (Loeb and Wasteneys, 1913; Inman, 1920; Caldwell, 1925; Bodine, 1933) in respiratory rates have been reported for animals when exposed to dehydrating hypertonic solutions.

In the present investigation the rates of dehydration and the resultant effects on the oxygen consumption of the eggs of the grasshopper, *Melanoplus differentialis*, have been studied.

MATERIALS AND METHODS

The embryology of this species has been closely followed by Slifer (1932*a*), while the changes in respiratory rate during development have been recently pointed out by Boell (1935). Briefly, at 25° C. the embryo develops regularly to the twentieth or twenty-first day, at which time a period of non-development (diapause) follows. During diapause, mitosis ceases and respiration and other activities are markedly lowered or depressed. If such non-developing eggs are placed at low temperatures (0° C.) for several months, and subsequently returned to 25° C., development is resumed, and hatching occurs about 20 days later.

Two methods were employed for dehydration: a hypertonic balanced salt solution (Belar) (Slifer, 1934) and a calcium chloride desiccator. The solution used was ten times isotonic strength, and will be known hereafter as a 10 N Belar. The eggs were not immersed directly in the solution, since in non-aërated or non-shaken solutions they soon deplete the oxygen supply and development ceases.[2] Eggs were therefore covered with a thin film of the solution in question and placed in a glass-covered vessel on filter paper saturated with the hypertonic medium. Both the walls and top of the vessel were lined with filter paper, wet with the same 10 N Belar; and the eggs were changed to a new vessel daily to prevent undue concentration by evaporation. Further details of dehydration methods appear in what follows.

The degree of water loss was determined by weighing the eggs on a chainomatic balance, sensitive to 0.1 mg. The water content of

[2] Unpublished observations.

similar eggs during development has been followed and discussed by Bodine (1929).

Oxygen consumption was determined by means of differential Barcroft micromanometers, using individual eggs in all cases.

RESULTS

THE EFFECT OF DEHYDRATION ON OXYGEN-CONSUMPTION RATE

Figure 1 represents graphically the oxygen consumption for post-diapause eggs[3] dehydrated in 10 N Belar to the indicated amounts.

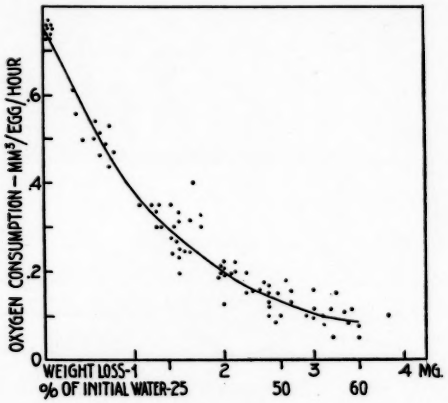

FIG. 1.—Oxygen consumption (cubic millimeters per egg per hour) of *Melanoplus differentialis* eggs at various levels of water loss (milligrams of weight loss and percentage of initial water). Each dot represents results for an individual egg. Temperature = 25° C.

From an inspection of this figure it will be noted that the rate of oxygen consumption drops rapidly during the first stages of water loss, tending to level off at about one-fifth of the normal undehydrated rate. This agrees with the previous results reported by Bodine (1933).

The effect of water recovery on the oxygen-consumption rates of dehydrated eggs is shown graphically for typical examples in Figure 2. If the water loss has not been excessive, recovery is slow and grad-

[3] "Postdiapause eggs" will be designated as those eggs in which the embryo has reached a stage in development about 12 days before hatching. The eyes are about midway between the two ends of the egg (Slifer, 1932*b*).

ual, both of water and of respiration (curves *A*, *B*, and *C*). If, however, water loss has proceeded beyond 2.5–3.0 mg., the egg gains water far in excess of that lost, sometimes regaining as much as 4 mg. above the initial weight. In such eggs, which occasionally swell enough to rupture the egg membranes, a "burst" of oxygen consumption invariably occurs (curves *D* and *E*). This "burst" sometimes reaches a height of 700 per cent of normal respiration, but it gradually drops off in a few days. In such eggs embryos are rarely found

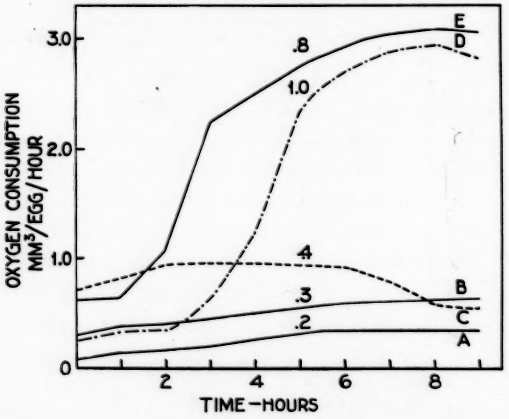

FIG. 2.—Effect of water recovery on oxygen-consumption rate of dehydrated postdiapause eggs. Oxygen consumption (cubic millimeters per egg per hour) plotted against time (hours). Number on curves indicates increase in weight (milligrams) above initial weight. Curves *A*, *B*, and *C*: individual eggs which lost 1.5–2.0 mg. during dehydration; *D* and *E*: eggs dehydrated 3.5 mg.

after swelling, although normal embryos were present at the beginning of the experiment, and shrunken but still visible embryos could be detected immediately after dehydration.

INDIVIDUAL VARIATION IN RATE OF DEHYDRATION

A large amount of variation was found in the rate at which water was lost. For example, in postdiapause eggs, after 24 hours' desiccation over $CaCl_2$, some eggs had not changed in weight, while others had lost as much as 82 per cent. Figure 3*P* shows graphically the extent of this variation. The eggs which fell into certain ranges in

respect to water (weight) loss are grouped together. Each egg was weighed, dried in the desiccator for 24 hours, and then re-weighed. Two per cent were found to have lost no weight; 11 per cent lost 0–0.5 mg.; 20 per cent lost 0.5–1.0 mg.; etc. One egg lost nearly 5 mg. It is interesting to compare this group with the diapause lot

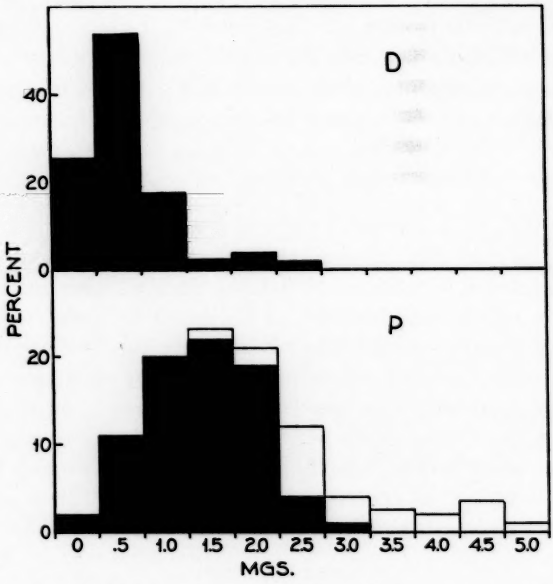

FIG. 3.—Variation in amount of weight decrease (desiccation) of diapause (*D*) and postdiapause (*P*) eggs exposed for 24 hours in $CaCL_2$ desiccator. Eggs, after exposure, were grouped as to weight loss: 0 mg., 0.0–0.5 mg., 0.5–1.0 mg., etc., and number in each group expressed as percentage of total number (300). Unshaded areas represent non-recovery.

(Fig. 3*D*). After the same length of drying time, 25 per cent of diapause eggs had lost no water; 54 per cent 0.0–0.5 mg.; etc. One can readily see that, while nearly all of the diapause eggs (80 per cent) had lost less than 0.5 mg. during the 24 hours' desiccation, 87 per cent of the postdiapause eggs had lost over 0.5 mg. during the same time. Although this is a wide spread, in the individuals of the older group (postdiapause) in particular, the rate of desiccation is more

rapid than in the diapause eggs. It should be mentioned that the postdiapause eggs are the most variable of any age, and that the diapause ones are the most uniform in regard to their individual differences in this respect.

This wide variation of water loss among individuals has been noted in other forms. Gunn (1933), using the cockroach, *Blatta orientalis*, found that from 5.5 to 32 per cent of the initial body weight could be lost in one day, even though the animals were subjected to the same environmental conditions. Ludwig (1936) mentions that certain larvae of the Japanese beetle are rapidly desiccated while others lose water very slowly. Larvae in the first instar may require from 23 to 142 hours to lose about 52 per cent of their weight.

THE VITAL LIMIT OF DESICCATION

After the eggs (Fig. 3) were weighed, they were placed on filter paper, moistened with water, for 2 days. Each was then examined to determine which had survived, as indicated by yolk engulfment. The black shaded portion (Fig. 3) indicates those which were developing, while those not recovering are shown by the unshaded part. Nearly all recovered if the loss was less than 2 mg., or 33 per cent of the weight. In the group which lost 2.0–2.5 mg., only one-third recovered; and it was apparently impossible for those which had lost more than 2.5 mg. to proceed with development. This is about 46 per cent of the total initial water content.

RATES OF DEHYDRATION

Figure 4 represents graphically the percentage of initial water content lost plotted against time of exposure to the 10 N Belar. Four indicated stages were used: 5- and 15-day prediapause, diapause, and postdiapause. Eggs of approximately the same weight were selected for each group. The temperature was kept at 25° C. Each curve is the mean of the dehydration curves for 300 eggs. The drying caused by the weighing had no appreciable effect on the rate of dehydration, as was demonstrated by weighing a group of postdiapause eggs every third day and noting that their water-loss rate did not vary significantly from that of the eggs weighed daily.

In terms of percentage of initial water lost, the developing eggs showed an increased rate of dehydration with increasing age. The

slowest group was the diapause lot. The limit of dehydration by 10 N Belar is about the same for all groups: 50–55 per cent of the initial water. This limit was ultimately reached by the diapause eggs, after about 6 weeks' exposure to the hypertonic solution.

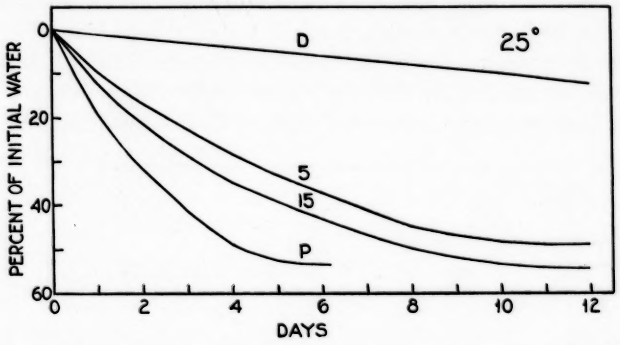

FIG. 4.—Percentage of initial water lost by eggs exposed to 10 N Belar. Temperature = 25° C. *5*, 5-day-old eggs. *15*, 15-day-old eggs. *D*, diapause eggs. *P*, postdiapause eggs.

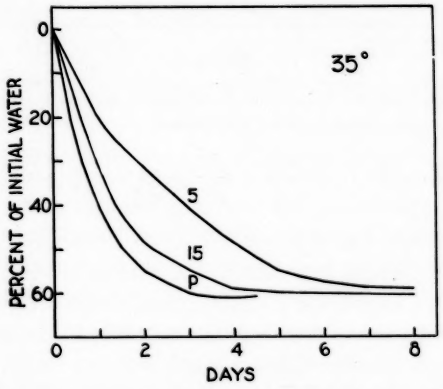

FIG. 5.—Same as Figure 4 except for temperature = 35° C

At 35° C. (Fig. 5), the rate of dehydration for all ages is higher, and the limit reached is about 60 per cent of the initial water. The curve for the rate of water loss of the diapause eggs is not shown since it followed almost exactly that for the postdiapause ones at

this temperature. It should be observed that eggs kept at temperatures as high as 35° C. have no apparent diapause.

The effect of three different temperatures on the rate of water loss by postdiapause eggs exposed to 10 N Belar is shown graphically in Figure 6. Since these eggs were all at the same morphological stage, they may be compared on the basis of actual weight loss. The 5° C. lot was eventually dehydrated to nearly the same level as the other groups. The Q_{10} for the dehydration rate between the interval 25°–35° C. is about 2; and between 5° and 25° C. it is much higher. This

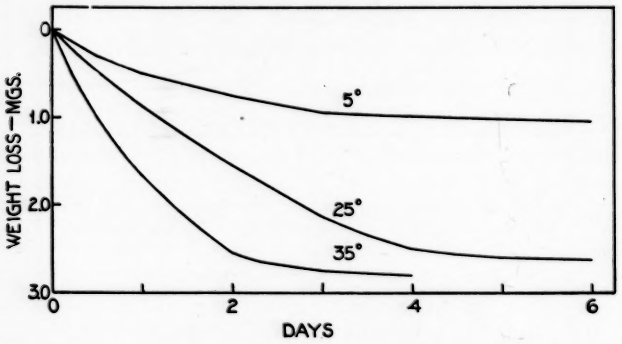

FIG. 6.—Weight loss (milligrams) of postdiapause eggs exposed to 10 N Belar at different temperatures, plotted against time (days).

type of variation in Q_{10} values is the usual result found when dealing with biological processes, but would be unusual for a process which might be considered as depending entirely on physical forces.

Figure 7 represents graphically the rate of drying of eggs over $CaCl_2$. From an inspection of this figure it is obvious that no qualitative difference exists between the results obtained by this dry desiccation and the wet (10 N Belar) method. Four- and 15-day eggs were quite close together in their rate of water loss; postdiapause ones were the most rapid. Diapause eggs were again the most resistant, but eventually (after about 40 days) lost 73 per cent of their initial water. The other groups lost between 70 and 76 per cent.

Obviously, in both methods of dehydration a great difference is shown between the rate of water loss from diapause and postdia-

pause eggs at 25° C. At 35° C., however, this difference disappears. Diapause embryos at that temperature become, developmentally, postdiapause embryos. Now, at 0° C., so far as can be detected, all development ceases. A comparison of the rate of desiccation at this temperature of the two types of eggs is shown in Fig. 8. No significant difference in the actual amounts of water lost (Fig. 8*B*) can be found at this temperature, where all demonstrable activity in both types of eggs is reduced to zero. This would seem to indicate

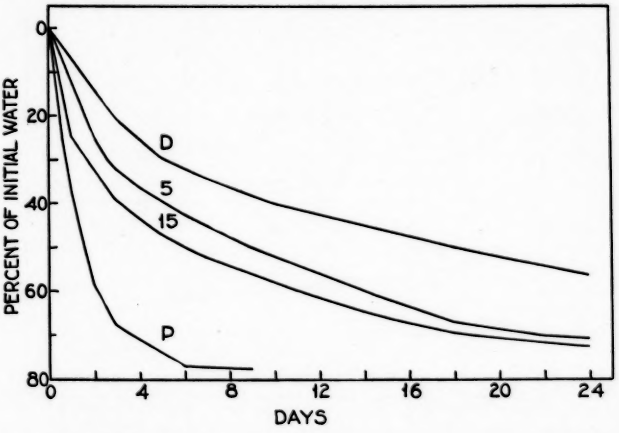

FIG. 7.—Percentage of initial water lost by exposure of eggs to desiccation over $CaCl_2$ plotted against time (days). Notations as in Figure 4. Temperature = 25° C.

that the difference in ability of the two types of eggs to withstand desiccation at developmental temperatures is related to the activity of the embryo. Accordingly, the rate of dehydration was determined for the isolated embryos. The methods used were those developed by Slifer (1934). Embryos of known stages were dissected out of the egg in isotonic Belar. After measuring the length of each embryo by means of an ocular micrometer, they were changed to 5 N Belar and measured every 5 minutes. Slifer (1934) found that postdiapause embryos in 5 N Belar shrunk to about 80 per cent of their initial length. This has been confirmed in the present observations (Fig. 9*P*). For diapause embryos (Fig. 9*D*), the same shrinkage occurs,

the rate of shrinking, if anything, being slightly more rapid than for the postdiapause embryos (Fig. 9*P*). Intact diapause eggs, therefore, are quite resistant to dehydration, while naked diapause embryos lose water just as rapidly as do diapause ones.

By centrifuging the grasshopper egg with the Beams ultracentrifuge, stratification of the contents occurs. The yolk, proteins, and remains of the embryo are clumped at one end of the egg, the remainder being filled with a clear, watery layer (Bodine and Boell,

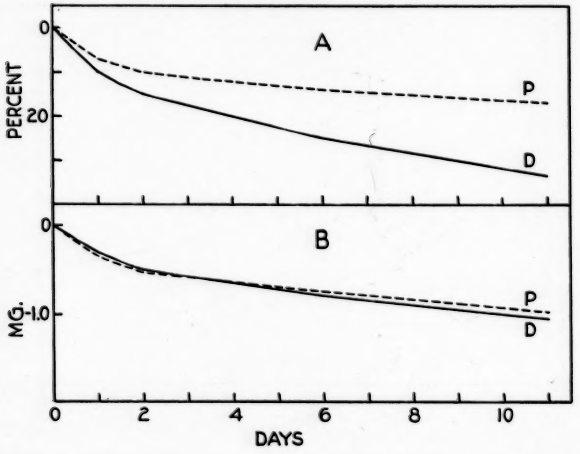

FIG. 8.—Percentage of initial water (*A*) and actual weight lost (*B*) by diapause (*D*) and postdiapause (*P*) eggs over $CaCl_2$ at 0° C. plotted against time (days).

1936). Eggs thus stratified have been employed to determine the rôle, if any, which the embryo itself plays in regulation of water loss by dehydration of the whole egg. The curves (Fig. 10*A*) still show the same qualitative rate difference between diapause and postdiapause eggs.

It has been shown that the respiration of centrifuged postdiapause eggs is depressed to a level approaching that of the diapause eggs. On the other hand, diapause eggs, treated in the same manner, respire at nearly their normal rate (Bodine and Boell, 1936). If the relationship between rate of desiccation and metabolic activity (Mellanby, 1932; Ludwig, 1936) is a simple function of respiratory

rate, the speed of desiccation of these centrifuged postdiapause eggs should then approach that of the diapause. Such, however, was not found to be the case.

Further evidence supporting the foregoing may be obtained by using intact eggs (if objection may be advanced to the centrifuge experiment on the grounds that such treatment is finally lethal). If postdiapause eggs be exposed to a mixture of 95 per cent carbon monoxide plus 5 per cent oxygen, respiration is depressed. Oxygen consumption of diapause eggs in the same gas mixture is little

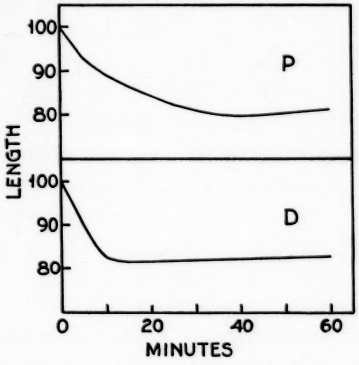

FIG. 9.—Changes in length (percentage) of isolated diapause (*D*) and postdiapause (*P*) embryos in 5 N Belar plotted against time (minutes). Each curve represents mean of 25 individuals.

affected (Bodine and Boell, 1934). Eggs of the two stages were placed in a $CaCl_2$ desiccator, which was then filled with this CO/O_2 mixture. The rate of water loss for each group was identical with that found in air.

The membranes surrounding the grasshopper egg are collectively known as the chorion and cuticle. The chorion may be removed from the egg after the cuticle has been formed (Slifer, 1932*b*). It was noticed that the chorions of postdiapause eggs were often cracked, sometimes peeling completely off the egg. In diapause and prediapause eggs the chorion is firmer—at least cracks or peelings are not often apparent. In order to test the function of the chorion in water loss, chorions were removed from a number of diapause and post-

diapause eggs and the eggs then dried as before (Fig. 10*b*). A qualitative difference in rate still exists between the two groups, although it is not quantitatively as large as for eggs with chorions untouched.

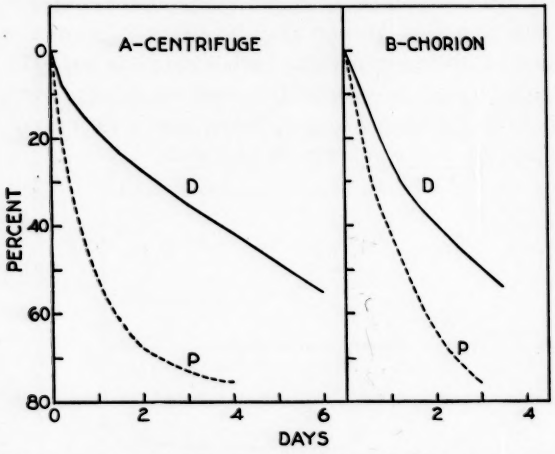

FIG. 10*A*.—Percentage of initial water lost by centrifuged diapause (*D*) and postdiapause (*P*) eggs plotted against time (days).

FIG. 10*B*.—Effect of removal of chorion on rate of water loss by diapause (*D*) and postdiapause (*P*) eggs.

Undoubtedly, the chorion serves for some protection, but it alone cannot account for the differences in water-loss rates of the two types of eggs.

DISCUSSION

It is not possible to explain the varying rates of water loss on the basis of stretching of the egg membranes. The egg gradually becomes heavier during its development, and this increase in weight is undoubtedly due to water intake (Bodine, 1929). It is rapid during prediapause, slowing down about the fifteenth to twentieth day of development. Weight remains relatively constant during diapause, increasing very slowly, if at all. Postdiapause eggs take up large quantities of water, about 2 mg. being the average increase in weight over a diapause egg. It is during these periods while water is normally being taken into the egg that it is lost most readily under

experimental dehydrating conditions. Diapause eggs, on the other hand, remain relatively constant in weight, take in but little water, and are very resistant to water loss. During the periods in which water is being imbibed, the egg increases in size and the membranes become stretched. Sometimes they are unable to stand so much swelling and rupture. The egg membranes, however, are not perfectly elastic, as is evident from the fact that postdiapause eggs commence to appear shrunken when they have lost 2 mg. or less of water. Diapause eggs, which contain about 2 mg. less water than postdiapause ones, are perfectly turgid; and such stretched membranes are undoubtedly thinner, as demonstrated by Jahn's (1936) work on capacitance. Being thinner, they may lose water more rapidly than before; and this might possibly explain the observation that 5-day, 15-day, and postdiapause eggs are successively less resistant to desiccation. Each group has respectively taken up more water, resulting in stretched and thinner membranes; but the diapause group, on the other hand, shows a striking exception. Here the water content is just as high, the membranes are stretched just as tight, and are as thin as in the 15-day group; but the rate of water loss is lower than for any of the developing groups. Furthermore, when diapause and postdiapause eggs are placed at 0° C., the rate of water loss in terms of actual weight loss is almost identical. In terms of percentage of initial water, the postdiapause egg is dehydrated at a slower rate than the diapause one—and this despite the fact that the postdiapause egg has more water to lose.

An extensive investigation of the properties of the membranes of the egg has recently been made by Jahn (1935*a*, 1935*b*, 1936). The only change in ionic permeability demonstrable by resistance measurements occurs with the laying-down of the cuticle, which takes place about the seventh day. No change was observed during diapause. There is, then, apparently no relationship between the ionic permeability properties of the membrane and water permeability.

A relationship between rate of water loss and metabolic activity has often been observed (see Hill, 1908; Hall, 1922; Ludwig, 1936). Ludwig's (1936) evidence is based on a more rapid loss of water during initial periods of desiccation, when the larvae are active. Later the larvae become inactive, and rate of water loss is less.

Further, in the pupal stages, desiccation is slowest. Respiratory rates are also low at this stage, as Ludwig (1931) has already shown. In the present study on the grasshopper egg, the same slow rate of desiccation was found for the diapause stage. The rate of desiccation apparently seems related to the general metabolic activity; but the situation, however, is not quite so simple. In isolated grasshopper embryos the rate of shrinking in 5 N Belar is as rapid for diapause as for postdiapause animals. In centrifuged eggs, internal organization is disrupted; yet the same qualitative difference in desiccation rate is evidenced as for non-centrifuged diapause and postdiapause eggs. Finally, diapause and postdiapause eggs lose water as rapidly in carbon monoxide–oxygen mixtures as in air. Such gas mixtures depress the respiratory rate of the postdiapause group but have little or no effect on the respiration of diapause eggs.

SUMMARY

1. The rate of water loss of *Melanoplus differentialis* eggs at different developmental stages has been determined. No qualitative difference in respect to rate was found between wet (with hypertonic solutions) and dry ($CaCl_2$) dehydration. The resistance to desiccation decreases with morphological age of developing eggs. Diapause eggs are most resistant.

2. The rate of desiccation does not seem to be closely associated with metabolic activity, as represented by that fraction of the respiration which is dependent on structure, or which may be depressed by certain CO/O_2 mixtures.

3. The oxygen consumption of dehydrated eggs decreases during water loss. In no case was an increase observed during dehydration.

4. The vital limit of desiccation of postdiapause eggs at 25° C. was found to be about 46 per cent of the initial water.

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VARIATIONS IN ACIDITY AND OXIDATION-REDUCTION POTENTIAL OF RODENT UTERINE FLUIDS[1, 2]

(Five figures)

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MANY morphological and physiological studies of the rodent's uterus have been made in the course of research into problems of embryology, reproduction, and endocrinology; yet little information concerning the fluids contained within the uterine lumen is available. This is surprising since the uterine fluids, affording the immediate environment for both egg and sperm within the uterine cavity, may affect directly the viability and implantation of the blastocysts as well as the viability and motility of the spermatozoa.

This investigation was undertaken to determine the hydrogen-ion concentration and oxidation-reduction potential of the uterine fluids *in situ* in the rat and mouse during the normal oestrus cycle, pseudopregnancy, and early pregnancy. These two characteristics of the uterine fluid were chosen for study, first because studies *in vitro* have demonstrated that the H-ion concentration and, to a lesser extent, the oxidation-reduction potential of media greatly influence the activity and longevity of spermatozoa and the development of eggs; and secondly because they can be determined quantitatively in living animals.

The literature describing the nature and origin of the uterine fluids in rodents is largely the record of observations made incidental

[1] Revision of a dissertation presented to the faculty of the Graduate School, Yale University, in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

[2] I wish to express my gratitude to Dr. J. S. Nicholas, under whose direction this investigation was begun and completed, for helpful advice and criticism and especially for his constant and kindly interest. I also wish to thank Dr. T. L. Jahn for valuable technical advice and for the use of his vacuum-tube potentiometer and other apparatus.

to histological studies of the uterus. An exception to this is the paper of Bond (1898), who investigated the secretory function of the uterus in the guinea pig and the rabbit and described the fluid collecting in a ligated uterine horn as a colorless, watery fluid, neutral or faintly alkaline in reaction, of very low specific gravity, containing some albumin and mucin and a remarkably large proportion of sodium chloride.

Emrys-Roberts (1905), and later Stockard and Papanicolaou (1917), described in the guinea pig abundant secretion of mucus by uterine epithelial cells during pro-oestrum.

Long and Evans (1922) and E. Allen, (1922), in their respective monographs on the oestrus cycles of the rat and mouse, state that toward the latter part of pro-oestrum the uterine cornua frequently become distended with a clear, watery, non-coagulable fluid, elaborated by the uterine glands.

The uterine fluids may be also partly of tubal and ovarian origin; but of the numerous papers dealing with the cyclic functional and structural changes in tubal epithelium, reference will be made only to the excellent review of Novak and Everett (1928), who were able to demonstrate changes in the human tube correlated with cyclic ovarian changes.

A number of histological and cytological studies of uterine mucosa in rats and mice, which have been made with modern staining techniques, reveal cyclic variations in the staining reaction of the uterine epithelium (Eisler, 1926; Glas, 1930; Reese, 1931).

The only recent investigation concerned chiefly with the nature of the uterine fluid is that of Aasland (1932), who attempted to lower the H-ion concentration of the uterine secretion in rabbits by producing a condition of acidosis in test animals. The pH determinations were made electrometrically with antimony electrodes inserted into the cervical canals of anesthetized rabbits. Aasland found that the pH of the cervical secretion of the experimental animals which did not differ significantly from that of the control animals varied from pH 7.85 to 8.40.

In an extensive investigation of the biology and physiology of spermatozoa and of vaginal secretions in cattle, Roemmele (1927), using a litmus-paper indicator, found that the H-ion concentration

of the vaginal fluids varied from pH 7.2–7.5 to pH 7.8–8.5. Roemmele was unable to establish a definite difference in the H-ion concentration of oestral, sterile, or pregnancy mucus.

The cyclic anatomical changes of the uterus in the rat and mouse during the normal oestrus cycle, pseudopregnancy, and early pregnancy have been carefully studied and correlated with the functional state of the ovary by Long and Evans (1922), E. Allen (1922), Parkes (1926, 1929), W. M. Allen (1931), and Clauberg (1931). These excellent studies have been used by the author in establishing the morphological condition of the uterus in respect to the various stages of the sexual cycle as determined by the vaginal-smear method.

MATERIALS AND METHODS

Animals.—The animals, mature female mice and rats, were studied under sodium amytal anesthesia. The rats were given the regular female dosage of 0.01 cc. of a 10 per cent aqueous solution of sodium amytal per 10 gm. of rat (Nicholas and Barron, 1932); but the dosage had to be slightly increased to produce complete anesthesia in those animals which were injected daily, as a tolerance for the amytal was acquired.

After experimenting on over a hundred animals, males and females, a dosage of 0.005 cc. of 4 per cent sodium amytal per gram of mouse was adopted as most satisfactory. Individual mice may react very differently to sodium amytal. A sufficient dosage for complete anesthesia in one animal may be inadequate in another, and lethal to a third. The decided difference in the reaction of males and females to sodium amytal, as found by Nicholas and Barron for the rat, does not hold for mice. In the mouse, sodium amytal is equally efficacious in males and females.

Apparatus.—The pH measurements were made by means of glass electrodes of special design which could be inserted through the vagina and os uteri into the uterine horn of the anesthetized animal without causing appreciable injury to the uterus or cervix. The glass electrodes were constructed from glass (Corning glass No. 015) of the composition recommended by MacInnes and Dole (1930). Two designs of electrodes were employed, a modification of the MacInnes and Dole type (see also Du Bois, 1932) and a capillary

type (Fig. 1*a*) similar to that described by Voegtlin and Kahler (1932). Electrodes of the MacInnes and Dole type were easily broken, and their performance when used in the uterus was less satisfactory than that of the capillary-tube type electrodes described below. They were discarded in favor of the capillary-tube electrode.

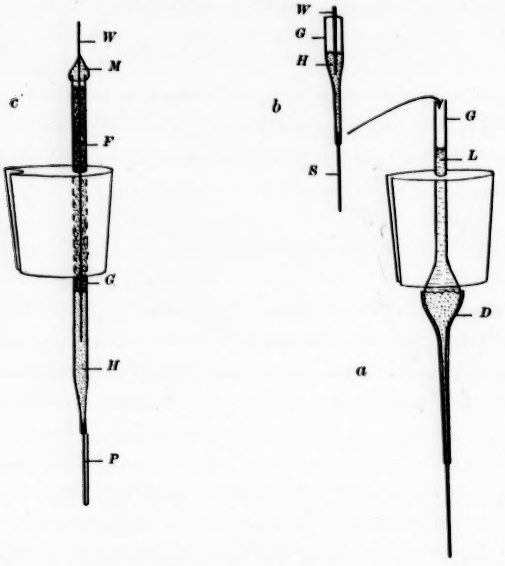

FIG. 1*a*, *b*, *c*.—Diagrams of electrodes used in making intra-uterine measurements: *a*, glass electrode; *b*, Ag/AgCl reference electrode; *c*, platinum electrode; *W*, copper wire; *M*, seal; *F*, cotton; *G*, glass tube; *H*, mercury; *P*, platinum; *S*, silver-coated platinum wire; *L*, M/10 HCl.

Capillary-tube electrodes which functioned satisfactorily electrically were too fragile for practical use in the uterus when they were made by drawing the capillary directly from glass tubing 5 mm. in diameter. To offset this difficulty a thin bulb, 15–20 mm. in diameter, was blown in a short section of the tubing, and the capillary was drawn from the bulb. The walls of the capillaries were not of uniform thickness but varied from 10 to 16 μ in the better electrodes.

The deviation film (see Kahler and De Eds, 1931) was eliminated by coating the bulb and capillary portion of these electrodes with

De Khotinsky cement. The electrodes were filled with M/10 HCl. Air bubbles were removed by suction and heating in a current of hot air. The electrodes were fitted into niched corks and kept in vials with the tips of the electrodes immersed in distilled water. The glass electrodes were then equipped with Ag/AgCl platinum-wire electrodes (Fig. 1*b*) and their electrode function tested. All glass electrodes showing high unstable asymmetry potentials were discarded.

The electron-tube potentiometer used in these experiments was built by Dr. T. L. Jahn (Jahn, 1935). The vacuum-tube potentiometer was built into a portable unit separate from the constant-temperature oven which housed the electrodes. Since the constant-temperature oven was copper-lined, all electrodes, animals, etc., were placed upon a false bottom, formed by a glass plate resting on paraffined corks. A Leeds and Northrup Type 2500*e* galvanometer was used in connection with a Type K potentiometer. Potentiometer readings were read to the nearest millivolt.

The platinum electrodes used to obtain the oxidation-reduction potentials were made from No. 26 platinum wire fused into a glass tube (Fig. 1*c*). Mercury was used in making electrical connection with the platinum wire. The best results were obtained with the electrode wire bent back on itself. This design both increased the active electrode surface and left the anterior end blunt, so that the delicate uterine tissues were less readily injured.

A silver-silver chloride electrode similar to the microelectrode described by Taylor (1925) was used as a leading-off and reference electrode. The Ag/AgCl reference electrode and the glass electrodes, when in use, were supported by individual stands (see Fig. 2). These stands had high insulating properties, were convenient to use, and were simply made by bending glass rods of appropriate lengths into the desired shape. The long end of the glass rod was fitted into a cork attached with sealing wax to a 6 cm. square of heavy plate glass to give the stand stability. The niched cork holding the leading-off electrode was attached to the supporting arm of such a stand by means of a rubber band.

Technique.—To hold the animal in position so that the ora uteri could be observed to facilitate the insertion of electrodes, a simple

stand was used (Fig. 2). The anesthetized animal was fastened to the stand by its tail, which was held to the sloping surface of the stand with adhesive tape. By holding the vagina expanded with a

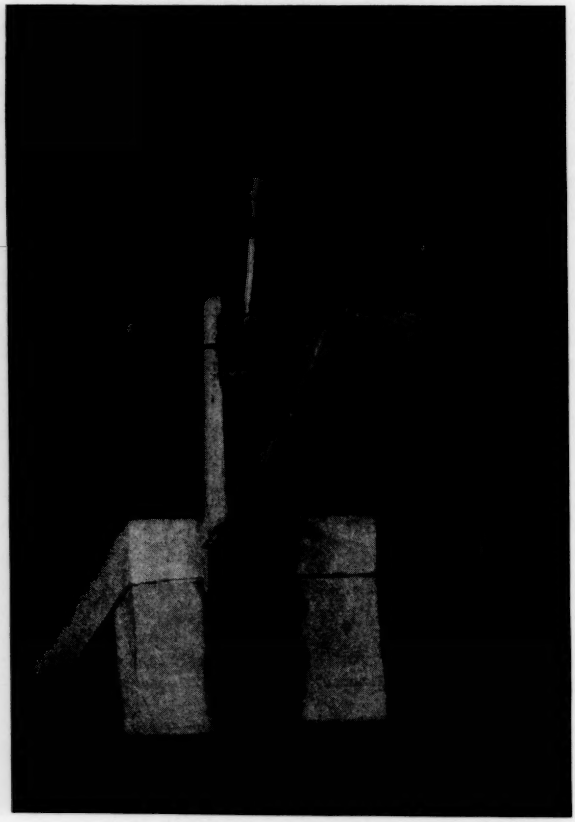

FIG. 2.—Anesthetized animal with glass electrode and reference electrode in position for taking pH readings.

small spring-brass speculum and a pair of curved forceps, and with a Nicholas lamp focused from above into the vaginal orifice, it was usually possible to recognize the ora of the right and left uterine horns as the electrode was inserted. The inserted electrode was held

in position by placing a rubber band around the supporting rod attached to the stand and the niched cork holding the electrode.

Before the electrodes were inserted, a vaginal smear was taken. The smears were fixed in ether-alcohol solution and stained with Erlich's haematoxylin, eosin, and water blue.

In making pH and Eh measurements the following routine was followed: The glass electrode was placed in the left uterine horn of an animal, and readings were taken for 10–15 minutes. The electrode was then removed, washed in distilled water, and inserted into the right horn. At times, especially if difficulty was met in entering the os of one side, determinations were made in one horn only. Oxidation-reduction potentials were taken in one cornu only, as it frequently took 30–60 minutes or more for the platinum-wire electrode to reach an equilibrium. The platinum electrodes were cleaned in chromic sulphuric acid cleaning mixture and were well washed in running water before use.

After an electrode had been inserted into the uterine horn, the animal was placed in the constant-temperature oven and the lead-off-reference electrode was placed on the animal. The practice that gave the most consistent and reliable results was to insert a piece of filter paper into the rectum of the animal, leaving the end of the paper projecting a few millimeters from the anus. The filter paper was then drenched with M/10 KCl until a drop of the KCl solution would remain on the paper. The tip of the reference electrode was placed in this drop of KCl solution. The potentiometer leads were then clipped to the electrodes and readings taken.

It was found advisable, and adopted as routine, in making all pH measurements in the uterus to check the observation before and after each determination by measuring the e.m.f. produced by the glass electrode in two standard buffer mixtures, one slightly more and one slightly less acid than the uterine pH. Using this method, the required pH can be interpolated, and errors due to the performance of the electrode are largely eliminated.

The drift and fluctuation in the potential developed by the glass electrodes in the uterine lumen was considerable at times, but the average drift was not over 6 mv. Although the pH values listed in the tables are calculated from readings which include the drift, the

average error probably does not exceed 0.05 pH. Voegtlin and Kahler (1932), who first used the capillary electrode glass in estimation of the H-ion concentration of tissues in living animals, found similar drifts; and they believe that with sufficient repetition of the measurements the average error of the method is not greater than 0.05 pH.

The MacInnes and Dole type of microelectrodes usually developed potentials indicating H-ion concentrations 0.1–0.3 pH less than a capillary-tube type of electrode inserted into the same uterine horn. This tendency is reflected in the low pH averages of Mouse Series II as compared to those of Mouse Series III.

The oxidation-reduction potential is established by a complex equilibrium of all the ions characterizing the system measured, and is influenced by the H-ion equilibrium as well as the specific oxidation-reduction equilibria. The oxidation-reduction potentials drifted from high positive values when first measured to more negative values as equilibrium was neared. This drift has been found by others in similar experiments with platinum electrodes, and is usually considered to be due to the presence of oxygen carried in on the electrode. Because fluctuations of 20–30 mv. in the platinum electrode potentials often occurred within a few minutes of each other, it was difficult to decide when an equilibrium had been reached. For this reason the final oxidation-reduction values listed in the tables may, in individual cases, be in error as much as 50 mv.

The standard buffer mixtures were Sørensen's phosphate buffer (Clark, 1928). The M/15 Na_2HPO_4 and M/15 KH_2PO_4 stock solutions were stored in well-stoppered 2-liter flasks equipped with soda-lime tubes, and the desired buffers were titrated fresh daily. The pH values of the phosphate buffer mixtures were checked to 0.03 pH by means of the quinhydrone electrode against standard M/10 HCl.

In classifying and referring vaginal smears to the various oestral stages, the following system of notation and classification has been adapted from Long and Evans (1922) and Allen (1922). A brief, generalized summary of the structural changes characterizing the uterus is included:[3]

[3] The letters "e" and "l" are occasionally used in connection with "I" and "II" to indicate that the smear has elements characteristic of the stage preceding or following it.

I.—Pro-oestrus smear: only nucleated epithelial cells. Uterine epithelium columnar and intact; marked distention of lumen and glands; leucocytes in stroma.

II.—Oestrus smear: only cornified cells not in large clumps or masses. Uterine epithelium cuboidal; degeneration begins in late II; glands and stroma much as in I.

III (e).—Early metoestrus smear: large clumped masses of cornified cells. Uterine epithelium undergoes vacuolar degeneration.

III (l).—Late metoestrus smear: cornified elements present, also leucocytes and nucleated epithelial cells. Uterine epithelium largely degenerate; no basement membrane, but repair of epithelium begins; glands show a minimum of function.

IV.—Dioestrus smear: leucocytes and usually stringy mucus with nucleated epithelial cells. Smear IV is found during the dioestrus interval of the normal cycle and throughout the period of pregnancy and pseudopregnancy.

EXPERIMENTAL SECTION

Experimental Series I.—Preliminary attempts to measure the H-ion concentration of uterine fluids colorimetrically were unsatisfactory and led to the adoption of the electrometric method. In these experiments the uterine horns were carefully excised and slipped over the electrodes. The uterine horn with the inserted electrode tip was then placed in a bath of Ringer's solution in the constant-temperature oven. Connection between the Ringer's solution and a beaker of M/10 KCl into which dipped the reference electrode was made by an agar-KCl bridge. Solution temperatures within the oven varied from 36° to 40° during the course of an experiment. pH readings were taken every 2 or 3 minutes for 5–60 minutes; then the glass electrode was replaced by a platinum electrode and Eh readings were obtained. Both horns of each uterus were similarly treated, but usually a delay of at least 10 minutes intervened between the initial pH readings of the right and left horns.

The results of these experiments, ten of which are tabulated in Table I, indicated that pH and Eh measurements of the fluids in the lumen of excised uteri are not reliable criteria of these characteristics in the living animal, for the initial pH and Eh readings were always followed by drifting electrode potentials. As the readings were continued in excised uteri, pH values became increasingly acid and the Eh values more negative.

To test the behavior of the glass electrode in the uterine lumen of a living animal, a mouse was anesthetized and the left uterine horn was exposed via a median ventral incision. A glass electrode was inserted into the lumen of the horn through an incision near the tubal-uterine junction. The animal was placed in the constant-temperature oven, and connection was established with the reference electrode by placing an agar-KCl bridge on the base of the uterus and

TABLE I

THE pH AND Eh VALUES OF FLUIDS IN LUMEN OF EXCISED MOUSE UTERI*

Mouse	Oestrus Stage	Horn	pH Values Initial-Final		Eh in mvs.	
A9M1	I	rt.	6.87 - .37	(35)	+169	(20)
		lft.	6.30 - .31	(30)	+148	(90)
A5M1	II	rt.	6.23	(0)		
		lft.	6.23 - .17	(20)	+164	(20)
A13M2	II	rt.	7.48 - 6.78	(15)	+111	(30)
		lft.	6.75 - .27	(38)	+198	(30)
A16M1	II	rt.	6.90 - .40	(60)	+193	(60)
		lft.	6.88 - .40	(45)	+250	(60)
A5M2	III (1)	rt.	6.39 - .30	(5)		
		lft.	6.58 - .57	(13)	+111	(75)
A11M1	IV	rt.	7.00 - .52	(15)		
		lft.	6.40 - .45	(20)	+195	(60)
A13M1	IV	rt.	6.88 - .43	(20)	+240	(60)
		lft.	6.47 - .31	(25)	+242	(30)
A13M3	IV	rt.	6.83 - .37	(34)	+179	(15)
		lft.	6.65 - .37	(15)	+183	(15)
A13M4	IV	rt.	6.93 - .40	(30)	+95	(21)
		lft	6.40 - .22	(30)	+88	(30)
A11M2	Preg. 50-60 hrs.	rt.	6.80 - .52	(25)		
		lft.	6.67 - .52	*(15)	+59	*(120)

* Numbers in parentheses represent time elapsed in minutes between initial and final readings.

moistening the uterine-bridge junction with M/10 KCl. The pH values obtained during 25 minutes' observation fluctuated between pH 7.13 and 7.40 but showed no tendency to fall to the acid values found in the excised uteri. The opposite horn was excised and, using the same electrode, pH readings were taken as before. The initial value was pH 6.45, and this soon dropped to pH 6.32. This experiment demonstrates that the H-ion concentration of uterine fluids in excised uteri is different from that which obtains in the living ani-

mal; so the following series of experiments were undertaken to investigate the pH and Eh of the fluids in the uteri of living animals.

Experimental Series II.—In this and the following series of experiments all observations were made on living animals. The pH and Eh measurements were made by inserting the electrodes into the uterine horns via the vaginal canal. The data obtained in the study of thirty mice are listed in Table II. The pH values found in this and the following tables under the heading "Initial" are not in all cases calculated from the first potentials obtained after the insertion of electrodes but at times from potentials recorded 3–5 minutes later. These values were used when the initial potentials fluctuated so widely as to appear unreliable.

The most alkaline values of the uterine fluids were obtained in animals in metoestrum, while the lowest pH values were found generally in uteri of animals in pro-oestrum. The low pH recorded for one horn of animal A30M6 in metoestrum is an exceptional case, perhaps the result of error in obtaining the pH. The pH of the uterine fluids of the animals in oestrus varied from a value similar to the average pro-oestrus pH to values as high as those obtained in the metoestrus group. The pH obtained in the uteri of the diosetrus animals averaged lower than that of the metoestrus animals and higher than that of the other groups. There were no very low or very high values in this group. The average pH value obtained in the pregnant animals was lower than that recorded for the dioestrus mice.

To test whether the means listed in the foregoing and following sections differ significantly, the data were treated by the method of Fisher (1930) for testing the significance of difference of means of small samples. A value of 0.05 or less for P has been considered as indicative of a significant difference of two means. By this standard, the pH means of Table II are significantly different for the pro-oestrus and oestrus group ($P=0.04$), for the dioestrus and pro-oestrus group ($P=0.01$), and for the oestrus and metoestrus group ($P=0.01$); but the differences of the pH means of the metoestrus and dioestrus groups and of the dioestrus and pregnant animals are of less significance ($P=0.14$ and 0.2).

The Eh means of all the groups, excepting the metoestrus group,

TABLE II

THE pH AND Eh VALUES OF UTERINE FLUIDS *in situ* AS FOUND IN 30 LIVING MICE*

Mouse	Oestrus Stage	pH Values Initial-Final	Drift in pH	Av. pH	Eh in mv.
A18M2	I	7.08 - 7.05 (55)	0.03	7.07	+185 (60)
A21M1	I	6.88 - 6.96 (15)	0.08	6.92	+195 (40)
A21M2	I	6.89 - 7.09 (30)	0.20	6.99	+146 (30)
A22M2	I	7.00 - 7.00 (15)	0.00	7.00	+184 (10)
A22M4	I	6.90 - 6.77 (10)	0.13	6.84	+289 (40)
A31M2	I	6.97 - 6.97 (25)	0.00	6.97	+233 (40)
For pH mean, σ=0.09 Average=			0.07	6.97	+205, σ= 46
A22M1	II	7.05 - 7.05 (10)	0.00	7.05	+210 (10)
A23M2	II	6.85 - 7.00 (20)	0.15	6.93	+165 (60)
A23M3	II	7.17 - 7.40 (40)	0.23	7.29	
A30M2	II	7.10 - 6.98 (10)	0.12	7.04	+222 (35)
A31M3	II	7.20 - 7.10	0.10	7.15	+205
A31M1	I (l)	7.13 - 7.20 (35)	0.07	7.17	+233 (40)
A30M8	I (l)	7.11 - 7.14 (15)	0.03	7.13	+228 (45)
For pH mean, σ=0.115 Average=			0.10	7.11	+211, σ= 25
A29M1	III (e)	7.20 - 7.35 (40)	0.15	7.28	
A30M3	III (l)	7.40 - 7.33 (45)	0.07	7.37	+185 (70)
A30M6°	III rt. (e) lft.	6.75 - 6.86 7.25 - 7.23 (60)	0.11 0.02	6.81 7.24	+139 (10)
A30M7	III (l)	7.57 - 7.43 (45)	0.14	7.50	+150 (77)
For pH mean, σ= 0.115 Average =			0.10	7.35	+158, σ=24
A18M1	IV	7.05 - 7.08 (20)	0.03	7.07	+205 (20)
A22M3	IV	7.23 - 7.30 (30)	0.07	7.27	+205 (30)
A23M1	IV	7.12 - 7.20 (60)	0.08	7.16	
A29M2	IV	7.30 - 7.38 (20)	0.08	7.34	
A29M5	IV	7.37 - 7.34 (20)	0.03	7.36	+241 (45)
A30M4	IV	6.95 - 6.90 (35)	0.05	6.93	+221 (25)
A31M4	IV	7.27 - 7.30 (15)	0.03	7.29	+153 (75)
For pH mean, σ= 0.158 Average =			0.05	7.20	+205, σ= 33
Preg. Mice					
A18M3	50-60 hr.	6.97 (30)		6.97	+206 (90)
A25M1	60 hr.	7.05 - 7.25 (20)	0.20	7.15	+256 (20)
A25M2	30 hr	7.07 - 7.13 (22)	0.06	7.10	+202 (33)
A29M3	50-60 hr.	7.05 - 7.13 (30)	0.08	7.09	+198 (45)
A20M1	40 hr.	7.10		7.10	
B1M2	72 hr.	7.20 - 7.13 (30)	0.07	7.17	+209 (25)
For pH mean, σ = 0.083 Average =			0.10	7.10	+214, σ= 24

*Numbers in parentheses indicate duration of readings in minutes
°pH average does not include value of rt. horn of A30M6.

$$\sigma = \sqrt{\frac{\Sigma(X-\bar{X})^2}{n-1}}$$

vary from +201 to +214 mv. and do not differ significantly. Only three Eh readings were obtained from animals showing metoestrus smears; but all three values are low, and the average (+158 mv.) differs significantly from the other means.

Experimental Series III.—H-ion determinations of the uterine fluids *in situ* were made daily for 8–17 days on eight mice. Oxidation-reduction potentials were also obtained, but not daily. The pH observations are summarized Figure 3, in Graphs I–VIII, and the Eh measurements in Table III. Because the glass electrodes did not maintain a definite equilibrium potential within the uterus, the pH values used in preparing the graphs were calculated from the average of two readings, the first taken soon after the insertion of the electrode, the other taken 10–15 minutes later. The average fluctuation corresponds to a deviation of less than ±0.04 pH from the mean value used in plotting the graphs.

The limits of variation of the observed pH of the normal uterine fluids in these animals were pH 6.93 and pH 7.70. The pH curves show decided fluctuations, which in general appear to be cyclic and correlated with the onset, culmination, and disappearance of cornified cells in the vagina. Minimum alkalinity in the uterine fluid was generally associated with pro-oestrum (average pH 7.09±0.13). This was followed by increasing alkalinity of the uterine fluid through oestrus (average pH 7.27±0.15), with the maximum alkalinity generally coinciding with the late cornified stages of the vaginal smear. The average maximum alkalinity was pH 7.43±0.13. These mean values differ significantly from each other.

The mean value correlated with all the early metoestrus smears was pH 7.24; but if the observations made during the periods of abnormal vaginal cornification in Mouse III and VI are omitted, the mean becomes the significant value, pH 7.35±0.17. The latter is probably the more accurate indication of the H-ion concentration of the fluid within a typical metoestrus uterus. A decreased alkalinity in the uterine fluid generally accompanied the advent of leucocytes into the vaginal smear. The pH curves during late metoestrum and dioestrum fluctuated erratically; but the average pH measured in late metoestrus uteri was similar to that recorded for dioestrus uteri, both being pH 7.24. The erratic fluctuations in the uterine pH

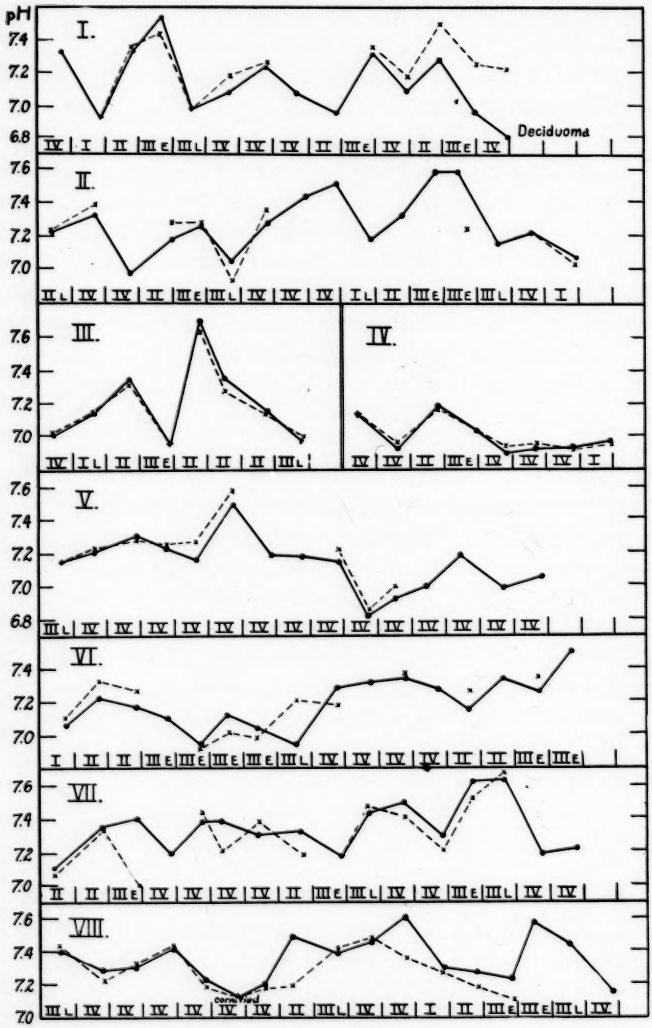

FIG. 3.—The H-ion concentration of the uterine contents of eight mice studied daily for 8–16 days. Vertical axis, pH values; horizontal axis, time in 24-hour intervals and type of vaginal smear at time of pH observation. Solid lines indicate left cornua; broken lines, right.

and abnormal vaginal smears observed in some of these animals may be due in a large measure to physiological disturbances arising from the daily anesthetization and insertion of electrodes.

Attention is drawn to the unexpected situation which developed in Mouse I. The vaginal-smear record indicated that three oestrus cycles occurred during the observations: yet on the fourteenth day of observation inspection of the uterus revealed the presence of a large deciduoma in the left cornu.

In relation to the results of the experiments on excised uteri, it is significant that the pH obtained in the uterus of Mouse VI, 2 min-

TABLE III

OXIDATION-REDUCTION POTENTIALS OBTAINED IN UTERINE HORNS OF MICE OF SERIES III

Oestrus Stage	I	II	IIIE	IIIL	IV
Eh	+275	+263	+165	+240	+215
"	+198	+141		+ 93	+234
"	+ 83			+192	+208
"					+282
"					+240
"					+265
"					+259
"					+222
Average Eh	+185	+202	+165	+175	+241
σ	97	86		75	26

utes after its death, was the acid value pH 6.65. This value is nearly ten times as acid as that observed in the same animal 16 hours before.

The oxidation-reduction potentials listed in Table III are similar to those found in the preceding experiments.

Experimental Series IV.—These experiments consist of daily pH and intermittent Eh observations made upon eight rats during a period of 16–17 days. The results of these observations are summarized in Figures 4 and 5, Graphs IX–XVI, and Table IV. The potentials developed by the glass electrodes within the rat uteri fluctuated relatively little. Although occasionally there were large

drifts up to 25 mv., the average was about 4 mv., which corresponds to a deviation of ±0.03 pH from the mean value used in plotting the graphs.

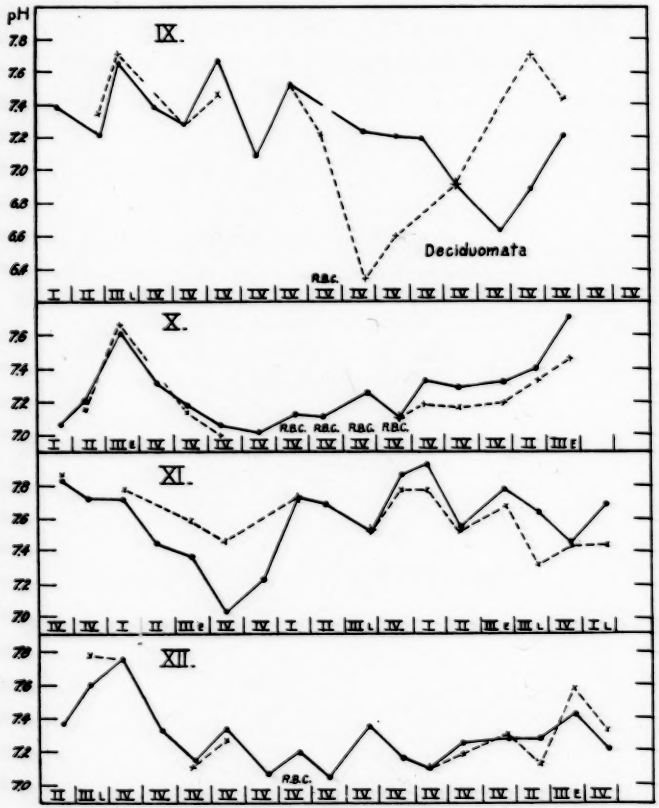

FIG. 4.—The H-ion concentration of the uterine contents of rats 1–4 studied daily for 17 days. *R.B.C.* indicates erythrocytes were present in vaginal smears; otherwise figure explanation the same as for Figure 3.

The H-ion concentration of the normal uterine fluids of the eight rats varied from pH 7.93 to 6.95. As in the mice the uterine fluids in the rats were characterized by a more acid nature at the onset of

oestrus than the fluids of the late oestrus or early metoestrus uterus. The record of Rat 2 closely approximates the average of all observations in this series. The results obtained in every animal are similar

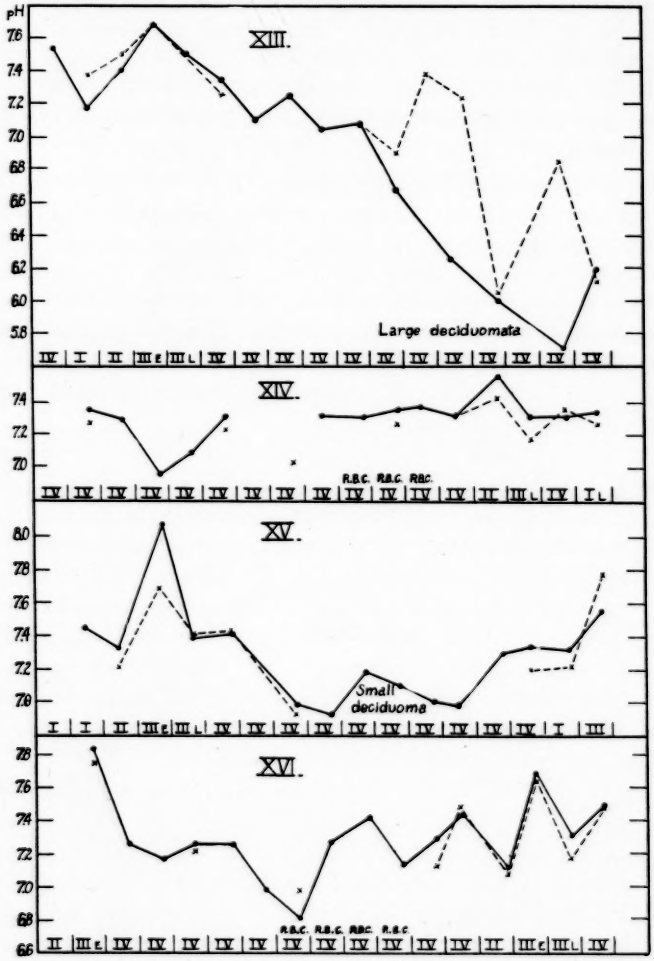

FIG. 5.—The H-ion concentration of the uterine contents of rats 7–10 studied daily for 17 days. Figure explanation the same as for Figures 3 and 4.

and consistent, except for the record of Rat 3, which will be considered separately since it offers several peculiarities.

As in the mouse series, the pH minimum preceding the increase in alkalinity of the uterine fluid was associated with either a stage IV, I, or at times a II smear. The average pH obtained in proestrus uterus was 7.29 ± 0.14, and in the oestrus uterus 7.31 ± 0.14. Readings taken 24 hours after oestrus readings, whether the vaginal smear was stage III (e) or III (l), showed a peak value in the alkaline direction.

The average alkalinity during stage III (e) was 7.69 ± 0.08; and during stage III (l), pH 7.43 ± 0.20. After the maximum alkalinity was attained, the uterine fluid rapidly became less alkaline, so that the average pH recorded during the first three days of stage IV was pH 7.26 ± 0.18. The average increase in acidity during this period was 0.5 pH. The foregoing data show that there is a marked significant increase in the alkalinity of the uterine fluid associated with the phenomena of oestrus, which is followed by an increased acidity in the following dioestrum.

The smear records show that six, and possibly seven, of the eight animals became pseudopregnant soon after the observations were begun. Although Rat 7 yielded a dioestrus smear for 13 days, it is possible that it was merely going through an extended interval period and not a period of pseudopregnancy. The pH obtained in the uterine lumen during the critical period 4–6 days after oestrus in the six pseudopregnant animals varied from 6.90 to 7.50, the average value being the significantly low pH 7.14 ± 0.17. A general average of the pH values obtained during the latter part of the pseudopregnancy cycle has little meaning in view of the widely different conditions occurring in the separate horns during this period.

Acid values from pH 6.80 to 5.72 were always associated with the presence of deciduomata in the uteri. The induction of the deciduomata was the result of accidental injury to the uterine mucosa during the "sensitive period" occurring 4–6 days after the induction of the condition of pseudopregnancy. Since the two uterine horns of an animal were not always investigated and injury was a matter of chance, it was possible for one cornu to develop deciduomata independently of the other cornu. This accounts for the great difference

between the pH curves of the two uterine horns of some animals. The large and erratic fluctuation in the pH curve of the right horn of Rat 6 on the sixteenth day of observation may have been caused by contamination of the electrode by blood and cellular débris which was present in the vagina. Small deciduomata were found in the anterior halves of both uterine horns of Rat 9 on the twelfth day of observation. Four days later the animal began a new oestrus cycle, and the alkalinity of the uterine lumen increased 0.6 pH.

The mean Eh values (Table IV) occurring in dioestrum and during the 4–6 and 7–14 days of pseudopregnancy differ significantly

TABLE IV

OXIDATION-REDUCTION POTENTIALS OBTAINED IN UTERINE HORNS OF RATS OF SERIES IV

Stage of Oestrus Cycle						Pseudopregnant		Deciduoma
Eh	I	II	III (e)	III (l)	IV	4 to 6	7 to 14 Days	
"	+257	+70		+248	+276	+265	+ 253	+ 173
"		+276		+195	+257	+267	+ 232	+ 176
"					+280	+356	+ 296	+ 134
"					+275	+288	+ 291	
"					+245	+295	+ 258	
"					+190	+315	+ 278	
"					+160	+308	+ 233	
"						+232	+ 245	
"							+ 210	
Average Eh	+257	+173		+222	+240	+291	+ 255	+ 161
6					47.2	31.3	28.8	23.4

($P = < 0.01$). The oxidation-reduction potentials obtained in the deciduomatous tissue average to the significantly low value of +161 mv. The values obtained during the fourth to sixth day of pseudopregnancy are all above +232 mv., and the values obtained during the latter half of pseudopregnancy are all above +210 mv.

Rat 3 was the only animal in this series which did not present a continuous dioestrus smear soon after the experiment began, and the pH curves of this animal are not consistent with those obtained in the other seven. Using the pH average for the other seven animals as a standard, the pH curve for this animal is abnormally high during stages IV and I. The average pH value during stage IV was pH

7.50 and during stage I pH 7.71, as compared to pH 7.26 and pH 7.29 for the other animals in this series. That this animal presents an atypical case is further supported by breeding experiments, which proved the animal to be sterile.

Experimental Series V.—The data of the pH and Eh observations made upon four rats in early pregnancy are summarized in Table V. In all cases the tubes were removed from these animals either before or directly after the observations listed here were made, and developing eggs were found in the two- and four-celled stages. The pH and

TABLE V

THE pH AND Eh VALUES OF UTERINE FLUIDS *in situ* IN RATS DURING EARLY PREGNANCY

Rat	Horn	pH Values Initial-Final	Drift in pH	Av. pH	Eh in mv.	Remarks
B13R1	lft.	7.46 - 7.49	0.03	7.48	+255	Preg. 48 hrs.
	rt.	7.50 - 7.54	0.04	7.52		
B13R2	lft.	7.42 - 7.40	0.02	7.41	+322	Preg. 48 hrs.
	rt.	7.44 - 7.42	0.02	7.43		
B15R1	lft.	7.37 - 7.45	0.08	7.41		Preg. 55 hrs.
	rt.	7.37 - 7.35	0.02	7.36		
B15R2	lft.	7.62 - 7.58	0.04	7.60		Preg. 55 hrs.
For pH mean, σ = 0.082 Average =			0.04	7.46	+289	

Eh observations were made as in the preceding experiments. The average H-ion concentration was similar to that obtained during late metoestrum in the preceding series.

DISCUSSION

Possible factors influencing pH and Eh fluctuations of the uterine fluids.—The maximum alkalinity of the uterine fluids was observed during late oestrus and early metoestrum, a period characterized by extensive vacuolar degeneration of the mucosa. The period of relative acidity of the uterine fluids during pro-oestrum and early oestrus is accompanied by rapid growth of the mucosa and active secretion by the uterine glands and epithelium. The secretions and exudations accompanying changes of the uterine mucosa and glands may account for the observed fluctuations in the acidity of the uterine fluids.

That a general condition of acidosis in an animal does not influence the H-ion concentration of uterine secretions has been demonstrated by Aasland (1932). However, the fact that the pH of the right and left uterine horns of Mouse I differed widely during the last 5 days of observation, while the pH curves for both horns fluctuated synchronously, raises the question whether the general metabolic state of the uterus may not be a factor influencing the H-ion concentration of the uterine fluids. It is significant, in this respect, that 24–40 hours prior to oestrus, the metabolic rate, as measured by the oxygen consumption of the uterus of the rat and mouse, is increased 300–400 per cent (Khayyal and Scott, 1931). It is possible that the increased activity of the mucosa cells during pro-oestrum, pseudopregnancy, and deciduoma formation may result in an increase in the CO_2 tension in the uterine lumen, which would tend to lower the pH of the uterine fluids at times coincident with the observed periods of increased acidity.

The development and growth of deciduomata is rapid, as is their degeneration. The acid condition and the low oxidation-reduction potential associated with the occurrence of deciduomata may arise from the pronounced metabolic activity of the decidual cells which are relatively isolated from the buffering and poising action of the circulating fluids.

The lack of complete correlation between the maxima and minima of the pH curves with the oestrus stages, as indicated by the vaginal smears, is believed to be the result of three factors: (1) the occurrence of some stages of the oestrus cycle between observations; (2) the animals were studied at varying times relative to the onset of the stage of oestrus indicated by the vaginal smear; (3) the vaginal smear is not an infallible index of the ovarian-uterine condition.

The pro-oestrus stage and the late metoestrus stage were most frequently missed, with the result that the low pH value preceding oestrus was, in such cases, associated with either a dioestrus smear or an early oestrus smear; and the pH minimum occurring after oestrus was associated with a stage IV smear. Different pH values of uterine fluids were often found in early and late periods of the same stage of oestrus. The importance of the time element and the lack of correlation of physiological oestrus with a definite "vaginal" oestrus is in-

dicated in that "heat," the "physiological" oestrus, may occur in pro-oestrum 3 hours before the appearance of cornified cells in the vagina or as late as 20 hours after their appearance (Long and Evans, 1922).

That the vaginal smear is not always an exact index of the ovarian uterine condition is supported by work of Hemingsen (1932), Clauberg (1931), and others. Hemingsen lists twenty-three cases where oestrus phenomena were exhibited in rats whose vaginal smear was dioestrus, late dioestrus, or late metoestrus. Clauberg, from a comparison of vaginal smears with histological studies of the uterus and ovary, concluded that the vaginal smear does not always afford an unquestionable index of the condition of the ovary, uterus, or even the vaginal epithelium itself.

In view of the possible lack of complete synchronization between the "vaginal oestrus," as indicated by the vaginal smear method, and the "uterine-ovarian oestrus," perhaps a clearer picture of the pH changes accompanying oestrus in the uterus may be had by considering the rise and fall of the pH curves without exact reference to the individual vaginal smears. If this is done, the average of the maximal pH values, usually occurring in early metoestrum in the rat series, is pH 7.67 ± 0.11 and in the mouse series pH 7.46 ± 0.14. The pH minima preceding oestrus average to pH 7.24 ± 0.10 in the rat and pH 7.11 ± 0.12 in the mouse, while the pH minima succeeding oestrus average to pH 7.21 ± 0.11 in the rat and pH 7.21 ± 0.13 in the mouse.

Pseudopregnancy induction.—Stimulation of the cervical canals of rats and mice during stages I, II, or III (e) of the oestrus cycle can be expected to interrupt the normal cycle and induce the condition of pseudopregnancy (Long and Evans, 1922; and Jongh, 1933). The smear records of the animals used in the present experiment show that this expectation was fulfilled in many cases.

It appears that only one mouse became pseudopregnant as the result of the rather vigorous stimulation of the cervical canals during the course of these observations. However, since a deciduoma was found in the left uterine horn of a second mouse, it must be concluded either that in this second animal the pseudopregnant condition developed or that a deciduoma was induced both to develop and

persist during normal oestrus cycles. It is the more likely that the condition of pseudopregnancy did not sufficiently suppress the cyclic rhythm of the ovaries (or ovary) to eliminate the periodic cornification of the vagina.

In the rat series, six definite cases of pseudopregnancy and a doubtful seventh developed, i.e., 75 per cent of the animals became pseudopregnant. This percentage is slightly above that obtained by Long and Evans (1922) and Meyer, Leonard, and Hisaw (1929) in unanesthetized animals. The latter authors report that lightly anesthetizing the animals with ether, nitrous oxide, or ethylene anesthesia decreased the percentage of pseudopregnancies resulting from artificial stimulation of the cervix to 10.4, 21.7, and 33.3 per cent. It is probable that in the difference of degree and duration of stimulation of the cervix lies the explanation of the discrepancy that exists between the results reported by Meyer *et al.* and those listed above.

Significance of pH and Eh findings in relation to spermatozoa and eggs.—Experiments *in vitro* on horse spermatozoa (Yamane and Kato, 1928) and on rabbit spermatozoa (Carter, 1932) have shown that mammalian sperm are very sensitive to small changes from the optimum H-ion concentration of the medium, which is between pH 7.2–7.4 for horse sperm and pH 7.9 for rabbit sperm. The alkaline nature of the uterine fluids during oestrus in the rat and mouse suggests that the uterine fluids may incite and favor the activity of spermatozoa *in utero*. The oxidizing nature of the uterine fluids (indicated by the Eh values obtained in the uterine lumen) probably also favors spermatozoan activity in the uterus.

In the modern concept of biological oxidations the oxidation-reduction potential of a medium plays an important rôle in cellular oxidation. The importance of the oxidation-reduction potential to developing sea-urchin eggs has been shown by Reiss and Vellinger (1929), who found that lowering the Eh of sea water to about +188 mv. (sea water = approximately +248 mv.) resulted in about 100 per cent cessation of development. The oxidation-reduction potentials of uterine fluids in the rat and mouse at the time of entrance of the egg into the uterus are well above those essential for the developing sea-urchin egg and indicate a system strongly oxidizing in action.

The pH curves of the pseudopregnant animals indicate an increase in the H-ion concentration of the uterine fluids at a time when the egg, in a normal pregnancy, would be free in the uterine lumen. This is especially true in the six pseudopregnant rats whose pH curves 4–6 days after oestrus show an average of pH 7.14 ± 0.17.

From Defrise's (1933) experiments on the culture of the rat egg *in vitro*, it appears that the increased acidity of the uterus in the early dioestrum and pseudopregnancy presents a more favorable environment for the developing egg than that of the oestrus uterus.

The period of implantation is coincident with the time when the endometrium is "sensitized" to deciduoma formation upon injury to the mucosa. It may be that the increased H-ion concentration observed in the uterine fluids during this period is either a necessary condition for the development of the decidua or merely a concomitant or resultant condition arising from the pronounced histo-physiological changes taking place at this time in the mucosa.

While the pH values obtained for the normal uterine fluids in the rat and mouse were higher than those of the acidified Ringer's solution used for oölemmal removal *in vitro* (Nicholas and Hall, 1934; Hall, 1935), the low pH obtained in the deciduomata indicates that the egg during normal development is subjected to an acid environment during implantation. As the decidua develops around the implanting egg and as the metabolic activities of the dividing blastocyst increase, the fluid bathing the blastocyst may become sufficiently acid to be a factor in the removal of the oölemma.

V. SUMMARY AND CONCLUSIONS

1. The uterine fluids, studied *in situ* in the living, uninjured rat and mouse, vary in H-ion concentration and oxidation-reduction potential during the normal oestrus cycle, pseudopregnancy, and early pregnancy.

2. The uterine fluids in the rat are in general more alkaline and show greater fluctuations during the oestrus cycle than those in the mouse.

3. In both the rat and mouse the occurrence of oestrus is associated with a decrease in the H-ion concentration of the uterine fluids, which reaches a maximum generally in early metoestrum. About 3

days after oestrus in the rat and slightly before this in the mouse, the H-ion concentration of the uterine fluids increases to a value approximately equal to that at pro-oestrum.

4. The oxidation-reduction potentials in the rat uterus were generally higher than those obtained in the mouse uterus. The evidence for cyclic variation in the oxidation-reduction potential during the oestrus cycle is not conclusive, although the values obtained during metoestrum were consistently low.

5. A decrease in the alkalinity of the uterine fluids was found during the fourth to sixth day of pseudopregnancy, which suggests that the decreased alkalinity is associated with the "decidual reaction" and that it may be of importance to the normal development and implantation of the egg.

6. The development and degeneration of deciduomata was characterized by a definite acidity of the uterine contents.

7. Two "regular" oestrus cycles, as indicated by vaginal smears, occurred in a mouse whose uterus contained a large deciduoma when examined on the eleventh day of "pseudopregnancy."

8. The acidity and the low oxidation-reduction potential which develop in the lumen of excised uteri demonstrate that such observations give an inaccurate and misleading conception of the conditions prevailing within the uterus of the living animal.

9. Cyclic variations in the glandular and metabolic activity of the uterine mucosa, correlated with the structural changes of the uterus, are suggested as possible factors influencing the variations in the H-ion concentration of the uterine fluid.

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ACTIVITY OF THE ADDUCTOR MUSCLE IN OYSTERS[1]

(Three figures)

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INVESTIGATIONS on the activity of the adductor muscle of lamellibranchs have been primarily from the point of view of feeding habits. In the oyster there is only one adductor muscle, morphologically the posterior, and this consists of a posterior rather crescent-shaped portion of striated muscle partially surrounding the more anterior plain muscle. The function of the striated portion is to cause the two valves to approach closer together. Its effect on the position of the shells is dynamic in contrast to that of the plain muscle, which serves to maintain any given position of the valves against the pull of the hinge ligament.

It was concluded by Kellogg (1915) that lamellibranchs feed only when the water pumped by the gills is relatively clear and that, when the water contains a large quantity of suspended matter, particles of silt and other material are altogether discarded and do not enter the digestive tract. Nelson (1921, 1922, 1923) made a study of the actual ingestion of food by the oyster under different conditions of turbidity of the water and reached the conclusion that the amount of silt in the water had little to do with the actual feeding process. He found that the adductor muscle contracted more frequently in water containing a large quantity of suspended material than in clear water, and used his findings in a study of the rate of feeding under different conditions. His method was to keep records of the shell movements and to estimate the rate of feeding from the turbidity of the water and the frequency of shell closures which result in the discarding of accumulated material.

Galtsoff (1928) called this method into question after a series of experiments in which he found that partial closures frequently occur without any obvious discharge of material. He also pointed out that contractions of the adductor muscle of a periodic nature were at

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times to be observed. However, the conclusions reached by Nelson concerning the rate of feeding of oysters at different temperatures are not greatly at variance with those obtained by Galtsoff using a more accurate method.

Recently Orton (1935) put forward the hypothesis that the function of the otherwise unexplained contractions of the striated portion of the adductor muscle is to relieve fatigue in the plain catch-muscle portion. This idea does not appear to be in harmony with what is known of the physiology of plain muscle, though it is not inconceivable that in the oyster plain muscle may require an occasional resting period. It was with the purpose of attempting to reach some conclusion as to the nature and function of the frequent partial closures of the oyster that the following observations were made.

MATERIAL AND METHODS

Already at hand were a great many kymograph records of shell movements of the Japanese oyster, *Ostrea gigas*, which had been made for different purposes. Not only do these records show the degree of openness of the shell and the movements due to the adductor muscle, but they show also the relative rate at which water is pumped. The method of recording the rate of flow of water has already been described (Hopkins, 1933, 1935). It consisted of a small cone attached to a system of levers and placed so that water from the cloaca struck the cone, causing a movement of the levers which recorded the relative rate of flow on the same kymograph papers on which shell movements were recorded. Some of the records were made at approximately constant temperature; others, at temperatures varying from about 2° C. to 30° C.

More direct study of the adductor muscle was made on small spat, 0.5–2.0 cm. in diameter, of *Ostrea lurida*, which had been caught on plane glass plates. The plates were put directly upon the stage of the microscope and the shells of the spat scraped clean so as to permit observation by transmitted light.

OBSERVATIONS

A study of the kymograph records indicates that partial closures due to contractions of the adductor muscle may be classified into

three groups according to the resulting discharge of water from the branchial and cloacal chambers. Characteristically, when an oyster is about to make a partial closure, the borders of the mantle arrange themselves so as to leave an opening between them in one particular locality while remaining otherwise close together, as described by Galtsoff (1930) for spawning by the female. In this manner, a strong current is ejected from a certain place, normally resulting in the discharge of waste or rejected material.

Some contractions involve a discharge primarily from the branchial chamber or some small portion of it. Other contractions are concerned almost entirely with the cloacal chamber, resulting frequently in the discharge of feces. A third type may be called non-

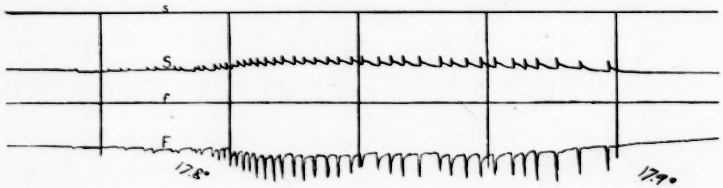

FIG. 1.—Reproduction of kymograph record showing a series of rhythmic cloacal expulsions. Upper record (*S*) is of shell movements as referred to the closed position (*s*); lower, relative rate of discharge of water (*F*) as compared to zero (*f*). Divided into 5-minute periods.

specific, the contractions being presumably due either to a general stimulus, such as vibrations or change in the intensity of the light, or to internal causes; and the borders of the mantle are not arranged to concentrate the discharge at any particular place.

A study of the kymograph records mentioned above clearly shows the marked distinction between the results of the different contractions. In cases in which the discharge was from the cloaca, the levers recording the rate of flow were violently disturbed; while if the discharge was from the branchial chamber, little or no effect was to be observed save that the rate of flow of water from the cloaca was suddenly reduced (Fig. 1). In the case of the non-specific contractions, a relatively slight disturbance of the lever recording rate of flow is to be observed.

Cloacal expulsions.—While contractions of the muscle occur in general with little apparent system, at times there occur periods of definitely rhythmic contractions which result in expulsions of water from the cloacal chamber. In Figure 1 is a portion of a kymograph record showing such a series. Typically, the contractions begin at a relatively slow rate, which very rapidly increases to a maximum and then slowly diminishes until the contractions cease. At the beginning of such a series, the contractions are small in amplitude, increasing gradually. A series of periodic expulsions may occupy only a few minutes or may be prolonged for an hour or more, as shown by Figures 2 and 3. One of the series shown graphically in Figure 3 is very regular; the other suggests a possible overlapping and fusion of two series. The figures represent extremes of variation in periodicity of this type of contraction.

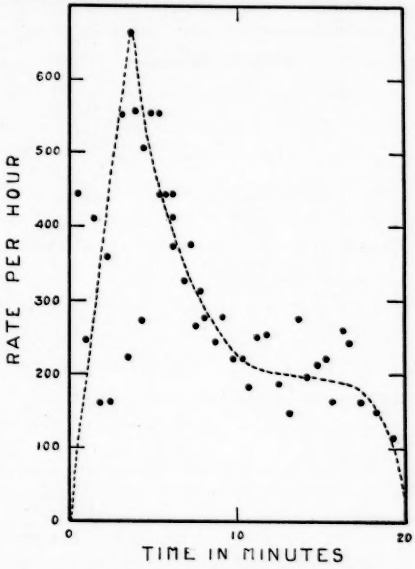

FIG. 2.—Frequency of rhythmic contractions of adductor muscle of *Ostrea gigas* determined from time between consecutive contractions. Same series as in Figure 1.

Apparently, the only important difference between the various series is in the duration of the activity at the most rapid rate. It is probable that the function of the series of rhythmic cloacal expulsions is the discharging of fecal material from the cloacal chamber. In a great many cases this has been actually observed, although such series may at times occur when there is no observable solid discharge. It is also true that defecation may take place without accompanying periodic cloacal expulsions. However, in cases of periodic contractions the borders of the mantle are so arranged as

to concentrate the discharge through the cloacal chamber. The form of the curves of Figures 2 and 3 describing these series of expulsions suggests what may be considered an internal origin rather than an external one, such as might be due to irritation by some foreign body. One may think of a series of nervous discharges arising in a portion of the digestive system through which waste material is passing.

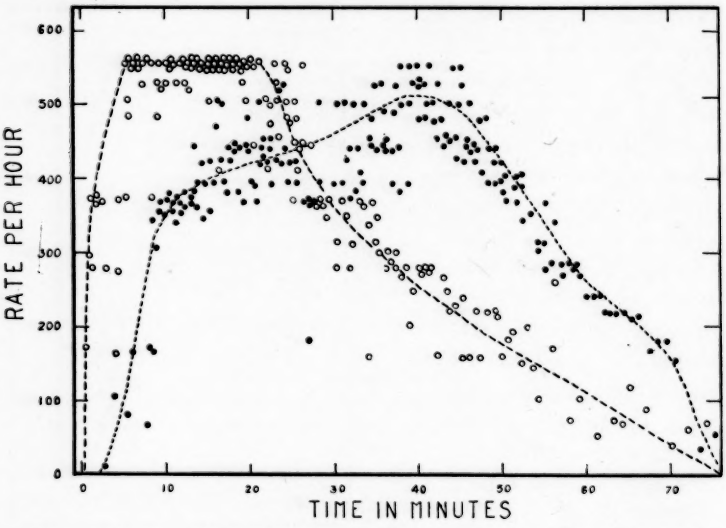

FIG. 3.—Frequency of rhythmic contractions of adductor muscle of *Ostrea gigas* determined from time between consecutive contractions. Two separate series.

Kymograph records of series of rhythmic partial closures, such as that shown in Figure 1, resemble those reproduced by Nelson (1922) and by Galtsoff (1930) of shell movements during discharge of eggs by *Ostrea virginica*. In the case of the spawning reaction, however, the discharge of eggs is from the branchial (mantle) chamber. Spawning by the male appears to involve no activity of the adductor muscle, for the sperms are released directly into the excurrent stream of water. That the periodic partial closures described above were not due to discharge of eggs is shown by the fact that during several experiments specimens which frequently exhibited such closures spawned as males, the two activities never coinciding.

Galtsoff (1930) also noted that adrenalin causes rhythmical contractions of the adductor muscle.

Other expulsions.—Although a great many kymograph records have been carefully measured and studied, there appears to be no obvious system in the occurrence of adductor contractions other than those described above. In some series of experiments the specimens remained for several days in the same water artificially circulated. In such cases there were few or no particles entering the water which might irritate the specimen, but yet occasionally there would be a sudden partial closure. In some cases these were non-specific, and in some they resulted in a concentration of discharged water from either the branchial or the cloacal chamber. The experiments were made in the laboratory, and it was often noticed that a sudden vibration would cause such a reaction. It is probable that most, if not all, of these expulsions are traceable to some external stimulation—either light, vibration, suspended matter in the water, or an accumulation of secreted mucus. In favor of such a view is the fact that the same specimen on consecutive days in the same water and under apparently almost identical conditions varies tremendously in the frequency of the contractions (Table I). Also, the records indicate that contractions are more frequent during a short time following a change of water which would contain more suspended matter. This is in accord with Nelson's conclusions. Furthermore, there appears to be no correlation between the degree of openness of the shell and the frequency of these contractions. Also, attempts to correlate the frequency of the contractions with the salinity of the water resulted in failure, save that a salinity below about 12 per thousand results in inhibition of the contractions as well as of the flow of water.

In experiments carried on with the temperature ranging from about 2° C. to over 30° C., the frequency of these non-specific contractions appears to have no direct relationship with temperature. On the other hand, periods of rhythmic cloacal expulsions definitely occur more frequently at temperatures above 15° C. than below, as would be expected if due to activity of the digestive system.

The adductor muscle of spat.—The spat shells of *Ostrea lurida* do not become greatly calcified until the diameter becomes over a centimeter. Spat caught upon glass plates were therefore excellent

material for direct observation. Studied by transmitted light with strong illumination the two sections of the adductor muscle are readily observable. When the spat is at rest, open and pumping water, the striated portion is very transparent while the plain muscle is relatively opaque. When the striated portion contracts, it becomes suddenly opaque and definitely darker than the plain muscle. As relaxation occurs, one readily sees that the all-or-nothing principle applies to this muscle, for the fibers return separately to transparency. As more of the fibers relax, the striated muscle takes on a splotched appearance, with some portions clear and others opaque.

TABLE I

FREQUENCY OF NON-PERIODIC CONTRACTIONS OF ADDUCTOR MUSCLE OF *Ostrea gigas* (SPECIMEN II) AT 17°–19° C.

Date 1932	Salinity (Parts per Thousand)	Total Time (Min.)	No. of Contractions	Av. No. of Contractions per Hour
11/28	28.87	185	41	13.3
11/29	22.70	385	128	20.0
11/30	22.70	335	40	7.2
12/1	28.74	355	90	15.2
12/2	28.74	390	24	3.7
12/3	28.74	160	9	3.4
12/4	25.08	325	157	29.0
12/5	25.08	250	19	4.6
12/7	28.94	310	16	3.1
12/8	17.85	145	18	7.4
12/9	17.85	180	22	7.3
12/12	17.83	265	15	3.4
12/13	17.83	235	19	4.9
12/14	17.83	120	10	5.0
12/15	17.83	80	11	8.3
12/16	17.83	135	6	2.7
12/17	17.83	130	23	10.6
12/19	27.27	265	35	7.9

Contraction appears to be brought about by synchronous action of all the fibers which take part in the process. The darkening is sudden, with no indication of progression. Relaxation, however, generally begins with the fibers adjacent to the inner border of the crescent-shaped muscle and passes across as an irregular wave. In the kymograph record reproduced (Fig. 1) it will be noted that the partial closures of the valves are quick, the record line being vertical.

Relaxation begins quickly but requires from a few seconds up to about a minute to become complete. Observations on the spat muscle demonstrate that this is due to the time required for all fibers of the striated muscle to relax.

Spat have been observed under the microscope in this manner for long periods of time, and the occasional contractions observed. If Orton's (1935) idea is correct that rhythmic contractions of the striated portion have the function of relieving fatigue in the plain muscle, one would expect to observe contraction of the striated portion as frequently when the oyster is closed as when open to any degree. However, in spite of many attempts, no such contraction has been observed when a specimen was closed. One would think that fatigue of the plain muscle would be at least as great when working against the entire strength of the hinge as when supporting only a small portion of this burden. Although one cannot state that contraction of the striated muscle does not actually result in relieving fatigue in the plain muscle, it would not appear to be entirely correct to say that such is a function of this activity.

DISCUSSION

Partial closures of the valves of the oyster are sometimes definitely periodic, sometimes apparently only occasional and unorganized. For hours at a time a specimen may remain open, pumping water vigorously, while the valves remain motionless. At other times partial closures occur more or less frequently. That at least some of the occasional closures are the result of external stimulation by vibration, change in the intensity of light, suspended matter in the water, and such factors, is well established, although it is not improbable that some of them may owe their origin to internal causes. The obvious function of these sudden partial closures is to discharge particles of silt, mucus, or other material from the surfaces of the mantle or gills; and one may observe the borders of the mantle become so arranged in advance of the closure as to direct most of the discharged water from some particular place. Even in cases in which there is no observable solid discharge, the directive action of the borders of the mantle may be seen.

On the other hand, there occur series of rhythmic closures the frequency of which varies according to a relatively definite system. In

all cases studied, these result in a discharge of water through the cloaca, often with accompanying observable defecation. It appears most probable from the nature of the rhythmicity that the origin of this activity is within the digestive system through which material is passing.

Partial closure of the valves is due to contraction of the striated component of the adductor muscle, while the smooth catch-muscle component maintains the valves in any given position. Physiological studies, which need not be reviewed here, have shown that catch-muscle expends little or no more energy when contracted than when relaxed. Orton (1935) questioned such conclusions by the hypothesis that rhythmic contractions of the striated component of the muscle of the oyster have the function of relieving fatigue in the catch-muscle. On theoretical grounds alone his idea would hardly appear to be tenable.

It was possible, however, to obtain some direct evidence on the matter by means of a microscopic study of small, relatively transparent oysters by transmitted light. Contractions of the striated portion of the muscle results in a sudden change from transparent to opaque; and while contractions were often observed when the valves were open, none could be seen when the valves were closed. Under the latter condition fatigue of the smooth component would presumably be at least as great as when the valves are open. Orton's hypothesis, which is an attempt to explain the origin of contractions of the striated muscle without direct observation on the muscle itself, does not appear to stand upon a very solid foundation in view of the observations described above as well as theoretical considerations.

The relaxation of the striated component of the adductor muscle furnishes an excellent example for direct observation of the application of the all-or-nothing principle in the individual relaxation of fibers.

SUMMARY

1. Contractions of the adductor muscle producing partial closure of the shell fall into three classes according to whether water is discharged (*a*) from the mantle chamber, (*b*) from the cloacal chamber, or (*c*) from both without direction.

2. Frequently there occur rhythmic partial closures resulting in expulsion from the cloaca. Such series are analyzed graphically; and it is considered probable that their function is the elimination of feces, although their origin appears to be internal.

3. Occasional contractions occur which may concentrate discharged water from either branchial or cloacal chamber. These are not obviously correlated either with the degree of openness of the valves, with temperature, or with salinity of the water, but are most frequent when the amount of suspended matter in the water is greatest. They appear to be due primarily to external stimulation, though some may have an internal origin.

4. By observing living spat by transmitted light the activity of the striated portion of the adductor muscle was studied. When contracted, it is opaque; when relaxed, transparent. Contraction is quick; relaxation, slow, involving a few fibers at a time. The application of the all-or-nothing principle was readily obvious.

5. In spat the striated muscle could never be observed to contract while the valves were closed, suggesting that Orton's (1935) theory that periodic contractions of this muscle serve to relieve fatigue in the plain muscle may not be well founded.

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A COMMON FACTOR IN PLANARIAN AND MAMMALIAN NUTRITION[1]

(Three figures)

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THE authors have carried on a prolonged series of investigations using the planarian worm to test the nutritional value of guinea-pig tissues (Bahrs and Wulzen, 1936). The guinea pigs were fed diets constructed for the study of the different vitamins, and their tissues became the sole diet of groups of planarian worms. It was found that liver, heart, and kidney derived from these guinea pigs produced, with only one exception, a dietary disease in planarians even though the recognized vitamins were adequately supplied in the diet given the animals furnishing the tissues. The one exception occurred when a supplement of green feed (the authors used kale and grass) had been included in the diet of the guinea pigs. The tissues from the animals receiving the green-feed supplement produced normal growth in the planarians, and the worms were entirely healthy. In certain experiments ample supplies of vitamin C were given the guinea pigs in the form of orange juice and tomato juice; but this in no way diminished the disease-producing power of the liver, kidney, and heart tissues furnished by those animals which did not receive kale or grass.

This protective factor for planarian worms seemed so definitely distinct from other recognized nutritional factors that it was given a name, factor *pl*. For convenience of reference this terminology is followed throughout the present paper.

Although the guinea pigs furnishing tissues which produced disease in planarians appeared normal during the course of the ex-

[1] Aided by a grant from the General Research Council of the Oregon State System of Higher Education.

periments, a period of about 2½ months, we thought it advisable to endeavor, by means of a more prolonged feeding period, to emphasize possible latent disturbances in the guinea pigs to a point where they might become overt.

Three prolonged series of experiments were completed on slightly different basal diets, Diets, 10, 11, and 12.

DIET 10

	Parts
Baked milk powder	25
Ground barley	42
Bran	23
Yeast*	5
Cod-liver oil	3
NaCl	1
$CaCO_3$	0.5
Ferric ammonia citrate	0.5
KI	0.002
(Given individually, 1 cc. of cod-liver oil, 2 drops viosterol, twice per week)	

DIET 11

	Parts
Baked milk powder	20
Ground oats	15
Ground barley	28
Bran	23
Yeast	5
Baked alfalfa meal	4
Cod-liver oil	2
NaCl, iodized	1
$CaCO_3$	1.5
Ferric ammonia citrate	0.5
(Viosterol, 1 drop per cubic centimeter of cod-liver oil)	

* We wish to express our sincere thanks to Mead Johnson and Company for generous supplies of yeast, cod-liver oil, and viosterol.

DIET 12

	Parts
Ground barley	30
Ground oats	27.75
Bran	16
Alfalfa, dried	20
Yeast	5
NaCl, iodized	1
Cod-liver oil	0.25
(Viosterol, 1 drop per cubic centimeter of cod-liver oil)	

FIRST SERIES OF EXPERIMENTS

Diet 10 was administered to six groups of guinea pigs. Supplements to this diet were given as follows: Group I, 14 females, 15 cc. fresh tomato juice per animal per day; Group II, 14 females, 20 g. fresh kale per animal per day; Group III, 7 females, 20 g. fresh kale and 15 cc. fresh tomato juice per animal per day; Group IV, 13 males, 15 cc. fresh orange juice per animal per day; Group V, 13 males, 20 g. fresh kale per animal per day; Group VI, 8 males, 20 g.

fresh kale and 15 cc. fresh orange juice per animal per day. All animals were bedded in straw and ate of it abundantly. Table I shows the average percentage gains of the groups. It is evident that the animals in the groups which received kale showed much higher percentage weight gains than did the animals in the groups which received no kale.

During the course of the experiment the animals in the groups receiving no green feed (Groups I and IV) gradually sickened and died, and the feeding continued until all the animals had succumbed —a period of 9 months. At the end of the experiment, all animals

TABLE I*

AVERAGE PERCENTAGE GAINS IN WEIGHT OF ANIMALS RECEIVING BASAL DIET 10, WITH OR WITHOUT ADDITION OF FRESH KALE

Group	No. of Animals	Sex	Diet Supplement	Av. Time (Weeks)	Percentage Av. Wt. Gain
I	14	♀	Tomato	20	65
II	14	♀	Kale	20	114
III	7	♀	Tomato and kale	20	115
IV	13	♂	Orange	16	38
V	13	♂	Kale	16	59
VI	8	♂	Orange and kale	16	57

* The lower gains of the groups of male animals as compared with the females were probably due to the fact that the males were very active and fought continually, while no fighting occurred among the groups of females.

in all groups which had received green feed (Groups II, III, V, and VI) were alive and in splendid condition. Since Group III received tomato juice and Group VI orange juice, it is probable that these juices had no intrinsic harmful effect on the animals.

In all cases where sickness occurred, a distinctive syndrome characterized the development and course of the disease. An early sign was the lowering of muscle tone so that the animals remained flaccid when handled. They developed difficulty in moving about the pen, because the hind limbs could not be manipulated well and became tangled in the straw. The righting time was considerably prolonged in these animals, and at length they could no longer right themselves when placed on their backs. The development of the scratch reflex was pronounced. In the normal controls any amount of tickling

failed to elicit the scratch reflex of the hind legs; but the subnormal animals first showed great restlessness under tickling along the back and sides and, as the disease progressed, they gave the scratch reflex at once on slight contact. In some individuals the reflex became so violent as to amount to convulsive seizures. There was also an involvement of the eyes, which was analyzed more carefully in connection with the second series of experiments and will be discussed later in the paper.

At a time when the disease was well developed, hemoglobin determinations were made on animals from five of the experimental groups. The Haskins-Sahli method was used in which 100 per cent is equivalent to 13.8 g. hemoglobin per 100 cc. blood. The results (in

TABLE II

BODY TEMPERATURE OF ANIMALS SHOWING SYMPTOMS OF FACTOR *pl* DEFICIENCY, COMPARED WITH THAT OF ANIMALS SUPPLIED FACTOR *pl* IN THE FORM OF FRESH KALE

Group	No. of Animals	Diet Supplement	Av. Body Temp. at 9:00 A.M.	Av. Body Temp. at 8:00 P.M.
II and V	15	Kale	100.7° F.	102.2° F.
IV	10	Orange	99.2	100.6
I	6	Tomato	99.8	100.7

percentage) were as follows: Group I, basal+tomato, 113, 124, average 118; Group II, basal+kale, 99, 100, average 99.5; Group III, basal+tomato+kale, 95, 99, average 97; Group IV, basal+orange, 115, 99, 100, 108, average 105.5; Group VI, basal+orange+kale, 98, 110, 94, 103, average 101.2. From these results it appears that the animals which received no kale and were showing marked signs of the deficiency disease manifested no diminution in hemoglobin.

We found that the body temperature of guinea pigs which showed definite symptoms of factor *pl* deficiency was lower than that of the controls. Table II shows the daily body temperature rhythm of animals from several of the experimental groups.

Guinea pigs manifesting symptoms of factor *pl* deficiency showed a marked fall in body temperature when subjected to a colder environment. Animals fed the same basal diet supplemented with fresh

kale suffered a very slight loss in body temperature when exposed to the same degree of cold. Table III illustrates this point.

Eventually the diseased animals could not move about the pen at all but lay on their sides with their legs extended. The joints were not swollen, nor was the hair rumpled; but the hair seemed thinner and coarser than in the healthy animals. After the animals were prostrate, death from inanition naturally supervened.

TABLE III

COMPARISON OF LOSSES IN BODY TEMPERATURE, WHEN EXPOSED TO COLD, OF ANIMALS SHOWING SYMPTOMS OF FACTOR *pl* DEFICIENCY AND ANIMALS SUPPLIED WITH FACTOR *pl* IN THE FORM OF FRESH KALE

No. of Animals	Diet Supplement	Time of Exposure to Cold (Min.)	Temp.	Average Loss in Body Temp. of Animals	
				Without *pl*	With *pl*
1	Orange	20	34° F.	3.1° F.	.....
2	Kale	20	34	.....	0.1° F.
2	Orange	30	34	3.1	.....
1	Kale	30	34	.....	2.0
2	Orange	30	40	2.1	.....
1	Tomato	30	40	5.1	.....
1	Tomato	20	40	4.2	.....
3	Kale	30	40	.....	0.46
3	Orange	20	45	2.6	.....
1	Kale	20	45	.....	0.3
2	Orange	30	45	2.7	.....
5	Kale	30	45	.....	0.6
2	Orange	20	48	3.3	.....
1	Kale	20	48	.....	0.1

We undertook to feed some of these individuals with a pipette to prevent starvation. The basal diet regularly used was made into a paste with water and was administered three times a day. The animals seemed very hungry and ate their food well. The orange and tomato juices were given as usual.

Those animals which were kept alive by pipette feeding and which did not receive kale gradually stiffened until both fore and hind limbs were thrust caudally in extreme extension. The toes showed a tendency to flex. There was some power of movement left in hip and shoulder joints. The legs were very strongly adducted, and the

posterior limbs could not have been spread apart without tearing the adductor muscles. The head was drawn back so that the occiput was pressed against the shoulder blades. It was impossible to press the head downward, and the whole body was so stiff as to be completely immobilized (see Figs. 1, 2, and 3). Even these very stiff animals ate well from pipettes, chewing and swallowing normally.

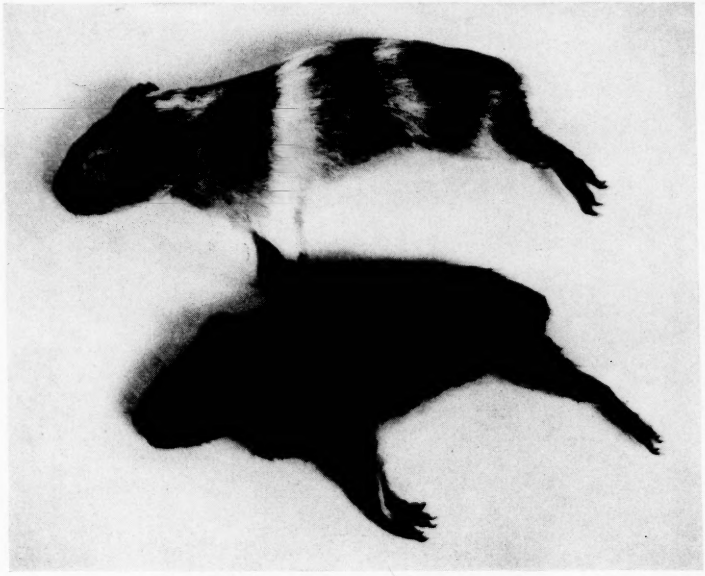

FIG. 1.—Live guinea pigs, fed Basal Diet 10 and orange juice for 6 months, until they became unable to move. They were then fed the same diet by hand for about 2 months. Their bodies were completely immobilized, the head and legs having the permanent forced positions shown.

The feces appeared normal also until the animals were about to die, when they became soft. For the last day or so before death the animals failed to eat well.

The following reflex was present: If light pressure toward the body was applied to the hind feet, the fore feet were thrown into a somewhat caudal position, and the muzzle moved slightly dorsad.

The eyes were open constantly, and no stimulation would make

them close. In the most developed cases they appeared opalescent. Hearing was very acute, and the animals twinged at any sharp sound when normal controls gave no sign. In one case when an injection was given in the posterior region of the abdomen, a cry of

FIG. 2.—A normal guinea pig lying on its back. Note spread of legs and position of head

pain was elicited. Terminally a coma developed, so that it was hard to rouse the animals to eat.

At autopsy, the liver often seemed hypertrophied and had a liquefied appearance, the hepatic lobules being indistinct. Others showed the hepatic lobules more distinct than in the normal animal. The liver was commonly mottled and often pale, giving the appearance of fatty degeneration. There were no conspicuous hemorrhages. The spleen appeared contracted and brownish or hypertrophied. The

ventricular tissue was soft and flabby. The skeletal muscles did not relax at all after death, the body maintaining the rigid position of life. There was extreme muscular atrophy. The muscles appeared grayish or yellowish white in color, of a waxy luster, and hard. In

FIG. 3.—One of the guinea pigs of Figure 1, lying on its back. Note the strong adduction and extension of the legs and the dorsally thrust head, in contrast to the normal positions shown in Figure 2.

portions they glistened because of the predominance of fibrous tissue. One case examined showed that in the adductor muscle mass a heavy tendinous band extended from the pubic bone to the inferior end of the linea aspera, with no intervening muscle. It was considerably shorter than the normal muscle would have been, so that the femur was strongly adducted and completely immobilized.

Skeletons prepared from diseased and control animals showed definite differences. The bones of the diseased animals were much thinner and more translucent than the controls. This was particularly true of the scapula, innominate bone, ribs, and vertebrae. The edges of the scapula and iliac bone were irregular and very rough. The transverse processes of the vertebrae were very thin and flattened. The spinal column could be cut across easily with scissors. Where the long bones of the controls were smooth, those of the diseased animals often felt finely granular. The translucent appearance of the bones and their strong odor suggested the presence of fat. The bones of the controls had no such appearance or smell.

In two cases we found it possible to restore a thoroughly sick animal to health by the addition of macerated kale to the diet, or kale leaves fed by hand when the animal became stronger. After the apparent recovery of one animal the kale was removed from the diet and the animal again developed the characteristic symptoms of the disease. When it was close to death the kale was restored, and the animal brightened considerably and developed a good appetite. It was kept alive for 2 months apparently in good health, but the stiffened joints were never restored to the normal condition and the animal was entirely helpless. It finally died of acute indigestion.

Another animal was given kale after it had stiffened, and the supplementary kale feeding was continued for 5 months. During this time there was a very gradual improvement from complete immobility and helplessness. The anterior end of the animal recovered almost entirely, so that the anterior limbs were quite limber and the head could be moved normally. The posterior limbs were permanently stiffened and adducted, so that the animal had to be set upon its feet in order to stand. When so placed, it could pivot about to reach its food; and it ate abundantly. It was finally killed because it showed no further signs of improvement.

SECOND SERIES OF EXPERIMENTS

Four groups of animals comprised the second series of experiments. They were given Basal Diet 11 with supplements as follows: Group VII, 13 females, 10 cc. fresh orange juice per animal per day; Group VIII, 10 males, 10 cc. fresh orange juice per animal per day;

Group IX, 7 females, 20 g. fresh kale per animal per day; Group X, 10 females, 6 cc. orange juice and 20 g. cooked kale per animal per day. This kale was soaked overnight in 2 per cent NaCl and was cooked in steam 1 hour. When the experiment had been in progress 3 months, the kale season was at an end and fresh alfalfa had to be substituted. The amount of alfalfa allowed each animal was 40 g.

The percentage gains in weight of the experimental groups at the fifteenth week of the experiment are shown in Table IV. It is evident

TABLE IV

AVERAGE PERCENTAGE GAINS IN WEIGHT OF THE ANIMALS GIVEN BASAL DIET 11 AND SUPPLEMENTS OVER A PERIOD OF 15 WEEKS

Group	No. of Animals	Sex	Diet Supplement	Percentage Av. Weight Gain
VII	13	♀	Orange juice	46
VIII	10	♂	Orange juice	38
IX	10	♀	Kale and alfalfa	72
X	10	♀	Cooked kale and alfalfa	63

that at this time the animals which received no kale had fallen in weight considerably below those which received kale; they were manifestly weak, and their weakness progressed steadily. At length, just as in the previous series of experiments, they became unable to move their hind legs through the bedding straw. As soon as each animal reached this point, it was segregated and given individual treatment, with the object of either emphasizing or curing the diseased condition. When it was found that the animals subjected to these treatments were not eating as they should, the basal diet which they were receiving was made into a mash with water and was fed to them twice a day with a medicine dropper. Some abbreviated protocols follow:

PROTOCOLS

Basal diet and orange juice without further supplement:

No. 301, female, Group VII. 4/30: Segregated because of stumbling in straw. 5/5: Wrists stiff; adductors are drawing legs together. 5/12: Legs stiff and thrust caudally. Helpless. Head drawn back onto shoulders. Still eats well from dropper but has no control over any part of body except mouth. 5/14: Dead.

Autopsy: Body rigid in all parts. Right heart much dilated. Liver enlarged, pale, and mottled. Lungs in good condition. Limb muscles hard and glistening in appearance, as if they were mostly tendon. Cutting away the muscles from the bones liberated the joints so that they could be flexed almost normally; thus the shortened muscles were the hindrance to joint mobility.

Addition of fresh alfalfa:

No. 277, male, Group VIII. 4/28: Segregated because of stumbling in straw. Fed by hand on basal diet mash. Given fresh alfalfa tips, which it could eat well. 5/3: Legs much better. Can now feed itself. 5/5: Apparently entirely cured. Dismissed.

No. 289, female, Group VII. 5/9: Segregated because of stumbling in straw. Given fresh alfalfa tips. 5/12: Can now run about pen almost normally, but is still weak and has not much appetite. 5/17: Very well in every way. Dismissed.

Addition of calcium:

No. 285, male, Group VIII. 5/7: Segregated because of stumbling in straw. Eyes show whitish exudate. Wrists stiff. Given injection of ½ cc. of 20 per cent calcium gluconate intramuscularly each day for 3 days; then 3 per cent of calcium lactate was added regularly to the basal mash. 5/14: Very weak; feeding by hand begun. Eating well. 5/17: Eyes very sore, filled with watery and milky exudate. Cornea opalescent. Legs very stiff. 5/22: Dead. Autopsy: Eyes have receded into socket and are suffused with exudate. Cornea dull. Eyeballs soft. Legs extremely stiff and strongly adducted. Liver pale and mottled and, when sectioned, appeared discolored around bile ducts. Auricles gorged with blood. Spleen shrunken and brownish. Formed feces in digestive tract. Had eaten well until a meal or two before death, when swallowing was difficult.

Addition of viosterol:

No. 223, male, Group VIII. 5/7: Segregated because of stumbling in straw. Given 15 drops of viosterol daily. 5/19: Can do nothing for itself. Legs stiffening. 5/22: Much worse but still able to eat. Eyes sinking back and quite watery. 5/23: Found dead. Autopsy: Legs moderately stiff at all joints. Much exudate, watery and milky, in eyes. Eyeballs soft. Liver pale and mottled; in section, discolored around bile ducts. Spleen shrunken and brownish. Kidney showed white infarcts. Formed feces in digestive tract. Had eaten from dropper up to very last. Lungs normal. Auricles gorged with blood.

Addition of cod-liver oil:

No. 314, female, Group VII. 5/9: Segregated because of stumbling in straw. Given 1 cc. cod-liver oil, containing 1 drop of viosterol, daily. 5/15: Became suddenly helpless and very sick. 5/17: Died. Autopsy: Much enlarged liver; spleen discolored. Formed feces in digestive tract. Heart distended with blood.

Basal diet without cod-liver oil or calcium:

No. 264, male, Group VIII. 5/22: Segregated because of stumbling in straw. Droopy but able to eat by itself. A new basal diet given, same as original Diet 11, except that cod-liver oil and $CaCO_3$ were omitted. 5/25: Now helpless, must be fed by hand but eats well. 5/28: Found dead. Autopsy: Eyes shrunken, with much whitish exudate. Eyeballs soft. Joints stiffened but not immobilized. Mottled liver; spleen discolored. Lungs normal. Auricles and right ventricle distended with blood.

Basal diet without cod-liver oil and with a large amount of yellow corn meal:

No. 250, female, Group VII. 5/22: Segregated because of stumbling in straw. Given the following basal diet: baked milk powder, 20 per cent; ground oats, 5 per cent; ground barley, 5 per cent; bran, 10 per cent; yellow corn meal, 48 per cent; yeast, 5 per cent; NaCl, 1 per cent; $CaCO_3$, 1.5 per cent; ferric ammonium citrate, 0.5 per cent; alfalfa meal, 4 per cent. 5/27: Now entirely helpless, must be fed by hand but eats well. 5/29: Found dead. Autopsy: Legs very stiff. Eyes watery; eyeballs soft. Liver pale and mottled, discolored around bile ducts. Spleen shrunken and brownish.

Addition of dried alfalfa ad lib.:

No. 303, male, Group VIII. 5/11: Segregated because of stumbling in straw. Was able to eat independently. Given all the dried alfalfa leaves it could eat in addition to the basal diet. 5/22: Still in good shape; eats well. To insure enough alfalfa, one feeding a day of alfalfa meal was made into a mash with water and was given by hand. 5/25: Entirely helpless but eats well. 6/2: Very weak; eyes contain exudate; hard to feed. 6/3: Found dead. Autopsy: Eyes suffused with exudate; eyeballs soft. Exudate from nose. Body very stiff; adductors strongly contracted. Liver abnormal; spleen contracted. Lungs hemorrhagic.

Addition of raw milk:

No. 237, male, Group VIII. 5/11: Segregated because of stumbling in straw. Given 30 cc. raw milk morning and evening, in addition to the ordinary basal diet. 5/17: Helpless; eats fairly well. 5/24: Very weak; death imminent; killed. Autopsy: Abnormal liver and white infarcts in kidney.

Addition of fresh carrot:

No. 269, female, Group VII. 5/25: Segregated because of stumbling in straw. Given 20 g. fresh, grated carrot daily. Ate it very well. 6/1: Can now run about easily. Scratch reflex elicited but with difficulty. Righting reaction slow. 7/9: Weight continued to drop until 6/11; since then has gone up steadily. Animal appears entirely cured. Killed for autopsy. Autopsy: Eyeballs soft. Heart flabby, decidedly softer than normal. Yellowish gray areas in liver. Spleen and kidneys normal.

Addition of dehydrated carrot:

No. 238, female, Group VII. 6/27: Segregated because of stumbling in straw. Given 5 g. dehydrated carrot daily, approximately equivalent to 20 g. raw carrot. Feeding continued until 7/14. No improvement; but animal retained the body weight it had before feeding of dry carrot began. Animal feeble and sensitive to tickling. Experiment discontinued.

Quantitative experiment to determine minimum protective level of fresh alfalfa:

No. 238, female, Group VII. 5/24: Segregated because of stumbling in straw. Given 2 g. fresh alfalfa tips daily. 6/11: Scratch reflex easily elicited. Righting reaction slow. 6/27: Condition generally worse. Two grams fresh alfalfa daily fails to maintain healthy condition in slightly diseased guinea pig.

No. 266, female, Group VII. 5/24: Segregated because of stumbling in straw. Extremely strong scratch reflex. Given 5 g. fresh alfalfa tips daily. 6/11: Scratch reflex elicited, but not so easily. Righting reaction slow; stumbles in straw. 7/11: Entirely recovered from stumbling. Very thin, and appearance not good. No scratch reflex. Killed for autopsy. Autopsy: Eyeballs soft. Liver has the characteristic smooth appearance, the hepatic lobules being indistinct.

No. 251, female, Group VII. 5/24: Segregated because of stumbling in straw. Given 10 g. fresh alfalfa tips daily. 6/11: Scratch reflex elicited with difficulty. Righting reaction normal. Eyes a little watery. 7/11: Weight gain rapid. Apparently entirely cured. Killed for autopsy. Autopsy: Eyeballs soft. Ventricular tissue softer than in control heart. Liver has smooth appearance.

No. 265, female, Group VII. 5/24: Segregated because of stumbling in straw. Give 15 g. fresh alfalfa tips daily. 6/5: Runs well in straw. Righting reaction almost normal in speed. No scratch reflex. 6/8: Dismissed as cured.

Summary of protocols.—No modification in the ultimate result was made by the substitution of Basal Diet 11 for Basal Diet 10. A continuation of the basal diet and orange juice led to the development of the symptoms already described and to death. If fresh alfalfa in liberal amounts was added to the diet of lightly diseased animals, the animals quickly regained apparent health. This indicates that factor *pl* is present in abundance in fresh alfalfa. A quantitative experiment showed that 10 g. and 15 g. of alfalfa tips a day were restorative, but 5 g. per day was only partially successful, and 2 g. per day allowed the animals to decline. The small amount of alfalfa necessary for protection indicates the vitamin-like character of factor *pl*. Dried alfalfa given a slightly affected animal prolonged its life for perhaps 2 weeks, but death finally followed the development of the usual syndrome. The drying process therefore decidedly reduced factor *pl* in the alfalfa.

Raw carrot, 20 g. per day, given a lightly affected animal, caused slow but certain improvement. After about 6 weeks the animal appeared cured, but at autopsy it showed signs of residual disease. Dehydrated carrot, 5 g. per day, given another sick animal, was unable to effect any improvement, although during the $2\frac{1}{2}$ weeks when it was being administered the animal was able to maintain its body weight. The indication is, therefore, that raw carrot contains factor *pl* but in much lower concentration than alfalfa, and also that drying greatly diminishes the amount of the factor in carrot.

The addition of extra calcium, viosterol, or cod-liver oil to the diet of lightly diseased animals caused no improvement. The animals sickened and died with the characteristic symptoms. Likewise the omission of cod-liver oil, viosterol, and calcium did not delay or modify the course of the disease after it had once become established. Replacing the cod-liver oil in the basal diet by large amounts of yellow corn meal was without remedial effect. Raw milk also was not remedial in the amount used. If factor *pl* is present in these products, it must be small in amount.

Cooked green feed.—Group X of the second series of experiments was given Basal Diet 11 and orange juice, and in addition 20 g. cooked kale per animal per day. This was changed to 40 g. cooked alfalfa after 3 months' feeding, because of the closing kale season. The green feed was left in 2 per cent NaCl overnight and was then cooked in steam for an hour. Table IV shows that at 15 weeks Group X had gained weight about equally with the animals of Group IX, which received a supplement of fresh green feed. This was true also after the experiment had continued 6 months. Externally the animals appeared to be in excellent condition and could not be distinguished from the controls. Nevertheless, autopsy showed definite signs of latent disturbance due to lack of factor *pl*. Of the 8 animals examined, 6 showed either the characteristic smooth appearance of the liver or flabby ventricular tissue or both. Eye changes were also found, which will be discussed in the next section. The controls from Group IX, killed at the same time, all had normal livers, hearts, and eyes.

Two guinea pigs suffering from factor *pl* deficiency to the extent that they could no longer walk in straw without stumbling were

given daily, in addition to their ordinary Basal Diet 11 and orange juice, a supplement of 15 g. alfalfa tips cooked as follows: The tips were put into boiling water in a closed container and were cooked 20 minutes. The feeding was continued for 22 days. The animals improved on the treatment and were able to run about, but they were very thin and their weight showed only slight increase. At the end of the experiment, the scratch reflex could still be easily elicited and the animals did not appear vigorous. They were autopsied, and both showed swollen pale livers in addition to eye changes.

Thus factor *pl* shows some resistance to careful cooking but is nevertheless considerably reduced in the process. Even 40 g. cooked alfalfa per animal per day was not sufficient to safeguard the tissues of healthy animals from the onset of early symptoms, and animals sufficiently advanced in the disease to show outward signs were helped only a little by the addition of 15 g. cooked alfalfa to their diet.

Eye involvement following lack of factor pl.—It was observed that animals suffering from deficiency of factor *pl* were apt to have flattened, almond-shaped eyes instead of the very round, bulging eye of the normal guinea pig. Animals succumbing to the disease exhibited eyeballs which were quite soft and sunken. The eyes were usually suffused with a thin watery and milky exudate. When removed, they were found to have become more spherical than the typical eye, through the disappearance of the corneal bulge and the equalization of the different diameters of the eye.

A comparison was made of the eyes of animals in Group X (supplement, 40 g. cooked alfalfa), with those of Group IX (supplement, 40 g. raw alfalfa). The animals were killed by ether, and the eyes removed from the orbit. After testing the softness of the eyeball, it was opened and the vitreous body carefully removed. This was placed on a glass plate and immediately compared with that of an animal from Group IX. Of the 8 animals from Group X thus examined, 7 were found to have vitreous humors definitely less gel-like than those from Group IX. Those of Group X exuded more liquid and held their shape less well than those of Group IX. Thus, eye differences between the animals of Groups IX and X were found, even though there was no difference in body weight, or, as far as could be seen, in general well-

being. However, at autopsy, the animals of Group X gave definite signs of liver and heart involvement.

Other examples of eye involvement follow. Animal No. 283, which had received 5 g. dehydrated carrot supplement and had become very stiff in the wrists, was examined shortly after death. The eyeballs were so soft that it was difficult to remove the musculature without cutting into them, and the vitreous bodies were very liquid.

Two animals, Nos. 282 and 242, which had received 15 g. cooked alfalfa daily from the time they first failed to get the hind legs through the bedding straw, were examined for eye condition. These animals were progressing very well, could right themselves, although more slowly than normally, and could get the hind legs through the straw. At autopsy, the vitreous humors of both were found to be more liquid and less firm than those of two control animals.

THIRD SERIES OF EXPERIMENTS

In the third series of experiments the effort was made to approximate a diet of natural foods by the elimination of the milk powder used in the previous series and the reduction of the cod-liver oil to 0.25 per cent. Dried alfalfa meal of the best quality to be obtained locally was used to guard against any possible deficiency of vitamin A resulting from the low level of cod-liver oil. The animals were divided into two groups. They were given Basal Diet 12 with supplements as follows: Group XI, 15 females, 6 cc. fresh orange juice per animal per day and 20 g. fresh alfalfa per animal per day. (The alfalfa was changed to kale during the winter season, but alfalfa was again given in the spring.) Group XII, 18 females, 10 cc. fresh orange juice per animal per day. The experiment continued for 38 weeks in this form. The average weight gain of Group XI was 141.8 per cent, and of Group XII was 114.7 per cent.

The hair of the animals in Group XI was thick and soft, while that of Group XII, receiving no green feed, was harsh and seemed thin. The muscles of the thigh were carefully palpated in all animals. With no exception the muscles of the animals receiving no green feed were harder and less bulky than those of the controls. Flexion at the carpal joint was tested to show the shortening of the extensor muscle group of the lower fore leg. The leg was held extended at

the elbow joint, and the attempt was made to flex the foot. This was easily accomplished in the animals which had received green feed; but in the animals which had had no green feed, a definite resistance was felt which kept the foot from being flexed at right angles to the lower leg. There was no exception to the development of this tension, but some individuals showed it more strongly than others. The righting reaction, which was practically instantaneous in the animals receiving green feed, was definitely slow in the other animals; and the scratch reflex, which seems never to be developed in normal animals, could be elicited in some of the animals which had no green feed.

DISCUSSION

Diets of natural foods, apparently adequate in the ordinary food constituents and recognized vitamins, were given groups of guinea pigs. These diets did not contain fresh green feed. The earliest indication obtained by us that the guinea pigs, so fed, were abnormal was that planarian worms fed certain tissues from these guinea pigs grew more slowly and ultimately developed a deficiency disease with a characteristic syndrome. When green feed in the form of fresh kale was given the guinea pigs, the worms receiving their tissues remained normal (Wulzen and Bahrs, 1935; and Bahrs and Wulzen, 1936). The time required to develop tissues disease-producing for planarians was 1 month, but at this time the guinea pigs themselves were entirely normal in appearance. However, when the guinea pigs received these same diets over a period of about 4 months, they developed a disease with a characteristic syndrome which was always fatal.

Symptoms suggestive of those obtained by us have been cited by workers in experimental scurvy. Smith (1927) found that, when life was prolonged for several months in a scorbutic guinea pig by feeding inadequate amounts of vitamin C, complete disuse of the posterior portion of the animal ensued. Pain sensibility remained in the portion affected. These symptoms were obtained by us when guinea pigs were given an abundant supply of vitamin C but were deprived of factor *pl*, as represented by fresh kale and alfalfa. It may be pointed out that the ordinary diet low in vitamin C is low or lacking in factor *pl*. The external signs of deficiency in factor *pl* take a con-

siderable time for complete development on supplemented scurvy-producing diets. While they might not be conspicuous in an animal dying quickly of acute scurvy, they could become predominant in an animal kept for a long time from acute scurvy by administration of inadequate amounts of vitamin C. Thus an animal suffering from "chronic scurvy" might be expected to show a combination of symptoms, some due to inadequate amounts of vitamin C but some also referable to lack or insufficiency of factor *pl*.

Skeletal musculature degeneration has been found in scorbutic animals by numerous investigators, and it is also one of the leading outward signs of lack of factor *pl*. Dalldorf (1929) has emphasized this as occurring in his scorbutic animals. Rinehart, Conner, and Mettier (1934) also repeatedly observed degenerative changes in the skeletal muscles of their guinea pigs suffering from chronic scurvy. Meyer and McCormick (1928) using guinea pigs for the study of scurvy, have noted the highly nervous condition of their animals and the development of permanent locomotor disabilities. Our animals showed extreme degeneration of the skeletal muscles, and their strongly developed scratch reflex indicated their high degree of nervous excitability. Even our completely immobilized animals showed higher sensitivity to sound than did the controls.

Muscular degeneration, produced entirely apart from scurvy, has been carefully studied by Goettsch and Papperheimer (1931) and by Madsen, McCay, and Maynard (1935). Both sets of investigators used apparently well-constructed diets containing adequate amounts of the recognized vitamins, vitamin C being given as orange juice or tomato juice. Goettsch and Papperheimer describe as the essential and primary lesion produced in their guinea pigs a degeneration of the skeletal muscle which they consider to be the ultimate cause of death. The muscles were atrophied, pale, and less translucent than normal. Their findings correlate with ours; but by hand feeding we have been able to maintain the life of our animals for considerable periods of time after they had become completely immobilized, and we are not certain as to the underlying cause of death. Madsen, McCay, and Maynard in their recent memoir (1935) have summarized their extensive work on the toxicity of cod-liver oil for Herbivora. Madsen (1936) has further elaborated on their findings;

and Turner, Meigs, and Converse (1936) have also obtained muscular dystrophy by means of cod-liver oil. The prevailing muscular degeneration which they describe is so similar to that of our guinea pigs that we consider the same causes operative in each case.

Soft, flabby heart musculature is a characteristic of guinea pigs suffering from *pl* deficiency. The liver is inclined to hypertrophy and may show either abnormally distinct lobulation or may be unusually smooth in appearance and have indistinct hepatic lobules. The liver is commonly mottled and pale with indication of pronounced fatty degeneration. The spleen may be either contracted and discolored or hypertrophied. Meyer and McCormick (1928) note similar liver changes in their scorbutic animals, and they have found degenerative changes in the cardiac musculature. Madsen, McCay, and Maynard (1935) list the same sort of liver and heart abnormalities in the Herbivora with which they have experimented.

Goettsch and Pappenheimer (1931) consider that the lesions described by them must be referred to some still unknown factor, and make passing reference to a possible protective effect of green foodstuffs. They offer arguments to demonstrate that their animals were free from latent scurvy. These we corroborate. We found our animals at autopsy free from gross hemorrhages, and the teeth were always firmly set in the jaw. Moreover, we gave one of our lightly diseased animals daily doses of 0.005 g. ascorbic acid for a week and could see no amelioration of the symptoms. Had as little as 5 g. of fresh alfalfa been used instead of the ascorbic acid, there would have been decided improvement. Madsen, Maynard, and McCay (1935) also consider that scurvy is not a complicating factor in their results.

According to Madsen and his co-workers, both cod-liver oil and some other element of their synthetic diet are causative factors in the production of the lesions cited by them. They find that the complete omission of a cod-liver oil product from the synthetic diet does not entirely eliminate the injury, and that the addition of cod-liver oil to a diet of natural foods produces the characteristic muscle lesions in their animals.

With diets of natural foods we have succeeded in producing degenerative muscular changes in guinea pigs when the amount of cod-liver oil in the diet was as low as 0.25 per cent. In contrast to this

we have obtained optimum growth and development in guinea pigs fed as high as 4 per cent cod-liver oil for the period of the experiment, 9 months. The animals were in prime condition, fat and sleek. They never showed a trace of stiffness. Whether or not slight muscular lesions occurred at the beginning of the experimental period we cannot say; but the effect, if any, must have been exceedingly small and of no ultimate consequence. We have accomplished this result by the use of certain specific foods which appear to contain a necessary accessory factor, i.e., fresh kale, fresh alfalfa, and fresh carrots. Dried alfalfa, cooked alfalfa, and dried carrots also contain the factor, but in much smaller quanitities. It seems to be present in very slight amounts in yellow corn meal and milk, and may be considered almost or entirely lacking in orange and tomato juice. Turner, Meigs, and Converse (1936) have shown that in rabbits a good quality of alfalfa hay added to a diet which was producing muscular dystrophy enabled animals to survive, and that growth and reproduction appeared only partially impaired by cod-liver oil feeding.

Though these studies on the nutrition of the guinea pig are entirely an outgrowth of our findings in planarian nutrition, we are of the opinion that the factor with which we are dealing is the same as that with which Goettsch and Pappenheimer (1931) and Madsen, McCay, and Maynard (1935) are concerned. We also think it is possible that those investigators who have worked with chronic scurvy may have developed a complex of symptoms in their animals, one bloc of which is referable to lack of this same factor, often rendered more apparent by an excess of cod-liver oil in the diet.

SUMMARY

1. A deficiency disease has been produced in planarian worms by feeding them tissues from guinea pigs given supposedly adequate diets in which vitamin C was furnished in the form of orange juice or tomato juice. When the guinea pigs received this diet over a period of several months, they also developed a deficiency disease, which invariably ended in death.

2. When the orange juice or tomato juice was replaced by a supplement of fresh alfalfa or kale, or these green feed supplements

were given in addition to the fruit juices, the guinea pigs remained in excellent condition throughout the experiment.

3. The gross morphology of the diseased animals showed extreme degeneration of the skeletal muscles, leading to immobilization of the entire body, degenerative changes in liver, heart, and kidneys, resorption of bone, and softened eyeballs.

4. The diseased animals differed from the controls in that they had lower body temperature, could not maintain their temperature as well in a cold environment, and showed higher nervous irritability.

5. Fresh kale and fresh alfalfa were found to contain an abundant supply of the active principle; fresh carrot, a moderate amount. Dehydrated alfalfa and carrot were found to have a low store of the active substance.

6. The modification of the basal diet to increase or eliminate cod-liver oil, viosterol, and calcium had no appreciable effect on the course of the disease already established. Neither did the addition of a plentiful supply of raw milk or a high proportion of yellow corn meal.

7. Carefully boiled alfalfa in liberal amounts retained enough of the active principle to maintain animals in an apparently healthy condition externally, but autopsy showed signs of disintegration of liver, heart, and eyeballs.

8. A basal diet containing 20 per cent dried alfalfa and 0.25 per cent cod-liver oil maintained the animals in normal condition for about a year, but the characteristic muscular dystrophy eventually developed.

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STUDIES ON THE CARBOHYDRATE METABOLISM IN PLANARIANS

(Seven figures)

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IN VIEW of the predominant rôle of the carbohydrate metabolism both in free-living worms (for example, *Lumbricus*, *Tubifex*) and parasitic ones (*Ascaris*, *Fasciola*, *Moniezia*), it seemed worth while to investigate the matter also in free-living flatworms. Because they were easily obtainable, I have chosen for this purpose two planarians, *Planaria torva* and *Dendrocoelum lacteum*. As far as I know, there have never been published quantitative observations along this line, although Gelei (1912) and Prenant (1922) have shown with morphological methods that the planarians are able to store glycogen. On the other hand, Hyman, Willier, and Rifenburgh (1924) doubt whether any carbohydrate is to be found in these animals. These contradictions may be due to the different species used by the different investigators.

MATERIAL AND METHODS

Most of the experiments were performed with *Planaria torva*, which was easy to find during the whole year in two small ponds near the laboratory. *Dendrocoelum lacteum* was collected in the same localities but was not obtainable as regularly and in the same quantity. Only fresh-collected animals were used. During the experiments they were kept in glass dishes in tap water without receiving food, and they were kept in the dark.

As everyone knows who has concerned himself with quantitative questions in planarians, it is difficult to weigh these worms accurately. I isolated them first with a hair pencil in a small, dry watch glass; removed, as far as possible, the last traces of water; and transferred the worms into a weighing glass, which I weighed rapidly with an accuracy of 1 mg. Then the worms were transferred

into the water of the experimental vessel, and the empty weighing glass was weighed again.

With a view to the different experimental objectives, the determinations of the carbohydrate content were made at different intervals. For each determination *Planaria torva* was used in groups of fifteen to forty animals (about 0.1–0.3 g.) and *Dendrocoelum lacteum* in groups of three to five animals (about 0.2 g.). Most of the carbohydrate determinations were made according to Simonovits' (1933) micro-modification of Pflüger's glycogen method, excepting that the sugar formed was determined according to Hagedorn and Jensen's method. In some experiments I used the method of Dische and Popper (1926), for total carbohydrate. In certain experiments the oxygen consumption was determined by means of a micro-modification of the Winkler method in general use in this laboratory. In others I used the new, more accurate oxygen titration described by Krogh (1935).

CARBOHYDRATE CONTENT OF *Planaria torva* IN DIFFERENT SEASONS

As the observations concerning the polysaccharide content of *Planaria torva* were made fairly regularly from February, 1935, until November, 1935, it is possible to relate the storage of polysaccharide to the seasons. Figure 1 represents the data concerning this question. They demonstrate clearly that *P. torva*, like various other animals (mussels and snails, for example), shows a regular seasonal cycle in the polysaccharide content. In the autumn the polysaccharide content becomes very high, with a mean value of about 3 per cent of the fresh substance. This corresponds approximately to 15 per cent of the dry substance (dry substance, about 20 per cent; cf. Buchanan, 1931). During the winter and early spring the polysaccharide content diminishes slowly, to about 1.4 per cent of the fresh substance at the end of April. It seems very probable that the storage of polysaccharide, which occurs in the autumn before the water becomes very cold, is biologically significant in providing the worms with a reserve of food substance on which they can live during the winter and spring until more food becomes available. It seems reasonable to assume that the planarians are less

active during the winter and that they will therefore not be as able to provide themselves with food, as they do during the warmer seasons. In the first days of May the polysaccharide content drops extremely rapidly, to the low value of about 0.5 per cent of the fresh substance; and it remains at this level during several weeks. This behavior is not easy to understand. My first thought, that it might be related to the sexual cycle, providing the eggs with a reserve of

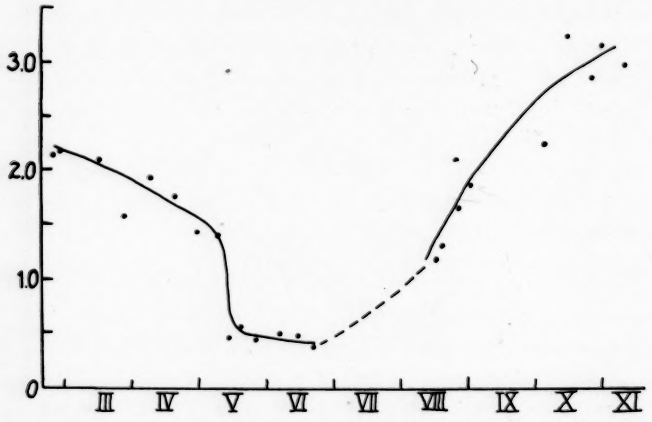

FIG. 1.—Variations of the polysaccharide content of *Planaria torva* at different seasons. Ordinates: polysaccharide content in percentage of the fresh substance; abscissas: months.

food, was not correct. An observation of the animal shows that they breed mainly during the months of May and June, when the polysaccharide content has already reached its minimum. The only relation between sexual life and polysaccharide content seems to be that the storage begins only when the breeding period has ended, an occurence which I found also in *Helix*. It would be interesting to investigate whether the changes in polysaccharide content are due principally or entirely to external factors, or, what seems more probable, also to internal factors. An investigation on animals regularly fed and kept at constant temperature would perhaps throw some light on this question.

CARBOHYDRATE CONSUMPTION DURING STARVATION

The investigations of Child (1919) and Hyman (1919, 1920) have shown that the oxygen consumption and the carbon dioxide output of planarians, which can be taken as a measure of the total metabolism, decrease during the first days of starvation, but that they increase later, even above the level of fed animals, when the oxygen consumption is referred to the weight of the animals on the day of the determination. In my experiments it was only possible to deal with the first phase, as after a starvation of about a week the carbohydrate content was so low that a further continuation seemed not to be advisable, since less than about 1 mg. is difficult to determine with sufficient accuracy.

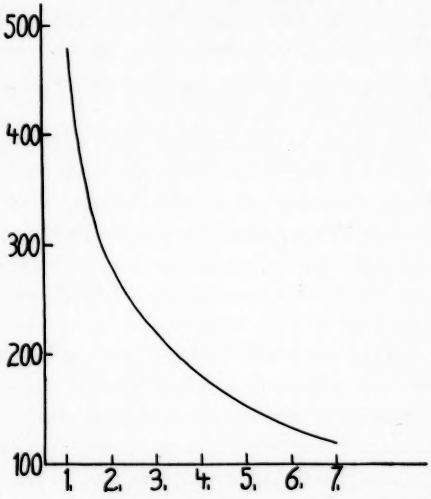

FIG. 2.—Rate of carbohydrate consumption in *Planaria torva* during the first week of starvation. Ordinates: milligrams of carbohydrate consumed by 100 g. of *Planaria torva;* abscissas: days of starvation.

The experiments have been performed at 25° C. Determinations of the polysaccharide content have been made at the beginning of the experiments and on the second, fourth, and seventh day of starvation. The data obtained in this way give the total amount of carbohydrate which has disappeared from the body on each respective day, compared with amount at the beginning of the experiment and referred to the initial weight of the worms. It is easy to deduce from this curve (Fig. 3) the curve of the daily polysaccharide consumption. The curve obtained in this way for *Planaria torva* is reproduced in Figure 2. It is similar to that of the above-mentioned authors for the total metabolism. The intensity of the carbohydrate consumption decreases during the first week of starvation. The rate

of decrease is greater during the first days, but diminishes later. During the first day, 100 g. of worms consume 0.48 g. of polysaccharide; by the seventh day, only 0.12 g. in 24 hours.

In addition to the experiments already mentioned, which were performed by the glycogen method, I have made a similar series of determinations by the method of Dische and Popper (1926) and obtained approximately the same curve. This shows clearly that during the first week of starvation polysaccharides are almost exclusively utilized. Otherwise the determinations by the method of Dische and Popper would have shown a greater decrease in the carbohydrate content.

It must be pointed out that these experiments were performed during February and March, when the worms had stored relatively large amounts of polysaccharide. Similar experiments performed during the summer months with planarians having only a small amount of polysaccharide demonstrate that in this case much less polysaccharide is consumed. I have carried out two experiments of this kind in June with worms containing, at the beginning, 0.75 per cent polysaccharide of the fresh substance. One hundred grams of worms consumed during 3 days, at 25° C., 0.43 g. of polysaccharide. The winter worms had consumed, during the same period and at the same temperature, 0.96 g. of polysaccharide. In comparative work on the carbohydrate metabolism of planarians it will therefore be necessary always to consider the seasons.

THE RELATION BETWEEN TEMPERATURE AND CARBOHYDRATE METABOLISM

The intensity of the carbohydrate metabolism both in *Planaria torva* and *Dendrocoelum lacteum* is, in a high degree, dependent upon the temperature. I investigated the consumption of polysaccharide in *Planaria torva* and that of total carbohydrate in *Dendrocoelum lacteum*. Three different temperatures were used: 25°, 14°, and 2.5° C., which were kept constant during the experiments within a range of ±1° C. The results are summarized in Figures 3 and 4. They are drawn to the same scale and show that the intensity of the carbohydrate metabolism is much higher in *Planaria torva* than in *Dendrocoelum lacteum*. During the first four days of starvation

the consumption (in milligrams of carbohydrate per 100 g. of worms) amounted to:

	Temperature (Centigrade)		
	25°	14°	2.5°
Planaria torva	1,100	480	260
Dendrocoelum lacteum	360	150	60

All these experiments were carried out during the winter and early spring. At this time the polysaccharide stored by *Planaria torva* was approximately 2 per cent of the fresh substance, the total

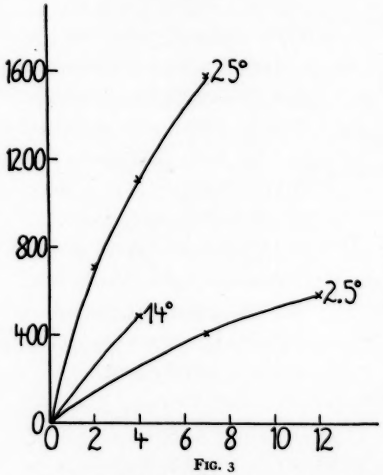

Fig. 3

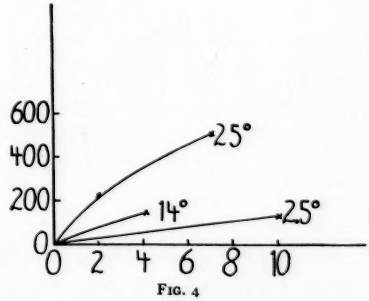

Fig. 4

Fig. 3.—Carbohydrate consumption in *Planaria torva* at different temperatures. Ordinates: milligrams of carbohydrate consumed by 100 g. of *Planaria torva;* abscissas: days of starvation. Temperature in degrees Centigrade.

Fig. 4.—Carbohydrate consumption in *Dendrocoelum lacteum* at different temperatures. Co-ordinates as in Figure 3.

carbohydrate by the method of Dische and Popper (1926) being approximately 2.5 per cent. *Dendrocoelum lacteum* had only 1 per cent of the total carbohydrate in the fresh body, of which only half

of the carbohydrates present at this time were polysaccharides determinable by Pflüger's method. Concerning the above-mentioned difference between the utilization of carbohydrate in winter and summer in *Planaria torva*, it seems reasonable to assume that in this case also the difference in the quantity of carbohydrates stored in the two species may explain the variation in the intensity of carbohydrate metabolism which is apparent at all temperatures used.

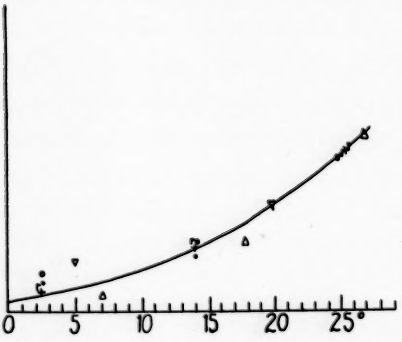

FIG. 5.—Projection of the carbohydrate consumption values at different temperatures of different animals on Krogh's normal curve. ●, ○, *Planaria torva*, values of the second and fourth days of starvation; +, ∠, *Dendrocoelum lacteum*, values of the second and fourth days of starvation; △, *Trypanosoma brucei;* ▽, *Iodamoeba bütschlii.*

A closer study of Figures 3 and 4 demonstrates that the increase in intensity in the carbohydrate metabolism with rising temperature does not conform to the rule of van't Hoff. I (1933) had formerly found the same in the case of the sugar metabolism of *Trypanosoma brucei*. The curve obtained at that time was more like the normal curve published by Krogh (1914) for the oxygen consumption of several species of animals. On the suggestion of Professor Krogh I have now projected on the normal curve the figures for the carbohydrate consumption of the first two and the first four days of starvation, as obtained from Figures 3 and 4, and have done the same with the previous data for the sugar metabolism of *T. brucei* and the glycogen consumption in the cyst of *Iodamoeba bütschlii*. The result of this procedure is shown in Figure 5. It is probable, but by no means certain, that Krogh's normal curve adequately expresses the relation between temperature and carbohydrate metabolism. At very low temperature the carbohydrate metabolism of these animals, and especially of the planarians, seems to be higher than the curve would indicate; but the difference may be due to experimental errors. This kind of experiment is more difficult

to perform in the case of carbohydrate metabolism than in that of oxygen consumption. In the latter it is possible to work on the same animal or the same group of animals at the different temperatures. Such a procedure is impossible in studying the carbohydrate metabolism, or at least in the species used in my experiments. It is obvious that the use of different animals must increase the error.

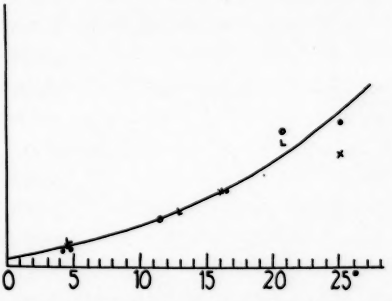

FIG. 6.—Projection of the oxygen consumption values at different temperatures of *Planaria torva* and *Dendrocoelum lacteum* on Krogh's normal curve. ●, ○, *Planaria torva*, first and second series; ×, ∠, *Dendrocoelum lacteum*, first and second series.

I have studied in four series of experiment—two on *Planaria torva* and two on *Dendrocoelum lacteum*—the relation of oxygen consumption to temperature in the same range as in the carbohydrate experiments, using also in this case three different temperatures in each series. The values obtained have again been projected on the normal curve (Fig. 6). We find here an irregular deviation from the curve, which indicates a better agreement than in the case of the carbohydrate metabolism. It seems very probable that Krogh's normal curve gives the best approximation for the relation of oxygen consumption to temperature in planarians also.

CARBOHYDRATE METABOLISM AND TOTAL METABOLISM

Several species of worms obtain the energy required for their vital processes mainly from carbohydrates. This is true, in the first place, of different parasites, as shown by investigations on the metabolism of *Ascaris* (Weinland, 1901; Schulte, 1917; von Brand, 1934; and others), *Fasciola* (Weinland and von Brand, 1926), and *Moniezia* (von Brand, 1933). The rate of consumption of carbohydrates is very high in these cases, as these organisms are not able to obtain appreciable amounts of free oxygen under biological conditions. Their type of metabolism is mainly a fermentation. From this pro-

cess much less energy can be derived than would be obtained from a total oxidation of the sugar, and the necessary consequence is a very intense rate of carbohydrate metabolism. But we know also, in free living worms, cases with high rates of carbohydrate consumption. Lesser (1908) has shown that the earthworm uses, at least in the first days of starvation, glycogen almost exclusively, with some protein. Much glycogen is also used by the polychaete worms *Owenia*, *Halla*, and *Spirographis* (von Brand, 1927). On the other hand, we know that the leech lives mainly on protein (Pütter, 1907, 1908; Lafargue and Fayemendy, 1932). These differences made it desirable to investigate the part which carbohydrate metabolism plays in the total metabolism of planarians. We have two ways to solve such a question: It is possible to determine the respiratory quotient and to draw conclusions from it concerning the level of sugar consumption in comparison with the total metabolism. I have made no determinations of this kind in planarians. I chose the second possible way: As mentioned above, direct analyses of the carbohydrate consumption were made. Furthermore, I have determined the oxygen consumption at 25° C. in two series each on *Planaria torva* and *Dendrocoelum lacteum*. These experiments extended over a period of 1 week, determinations being carried out in single periods of 24–48 hours. For both species a curve results which is representative of the total metabolism. Since we know the rate of carbohydrate consumption, it is possible to calculate the amount

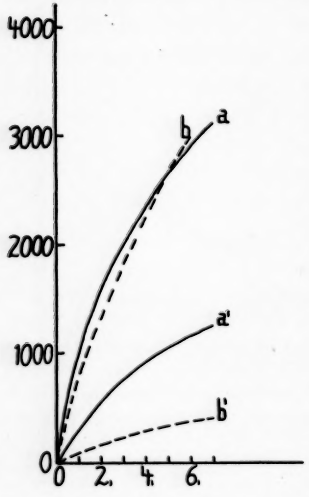

FIG. 7.—Total oxygen consumption of *Planaria torva* and *Dendrocoelum lacteum* at 25° C. and oxygen required for the sugar oxidation. Ordinates: cubic centimeters of oxygen consumed by 100 g. of animal; abscissas: day of starvation. *a*, *b*, total oxygen consumed by 100 g. of *Planaria torva:* (*a*) by 100 g. of *Dendrocoelum lacteum;* (*b*) *a'*, *b'*, oxygen required for the oxidation of sugar by 100 g. of *Planaria torva* (*a'*); by 100 g. of *Dendrocoelum lacteum* (*b'*).

of oxygen required for the oxidation of the sugar. We have to assume that the sugar is completely oxidized. This is not strictly proved, but is very probable, as Child (1919) has shown that the only acid produced by planarians is carbon dioxide. From our knowledge of the metabolism of other worms it is reasonable to assume that an incomplete oxidation of sugar in planarians would also lead to the formation of other acids.

Figure 7 shows the curves both for *Planaria torva* and *Dendrocoelum lacteum*. We can draw from them mainly two conclusions: First, the total rate of oxygen consumption of the two species is similar. It is relatively high but is in approximate accordance with those published by other authors (Hyman, 1919, 1920; and Allen, 1919) for other species of planarians. Second, the curves demonstrate clearly—which is somewhat amazing—that the proportion between total oxygen consumption and oxygen required for the oxidation of the carbohydrates remains practically the same during the single days of the first week of starvation. The proportion is, in the case of *Planaria torva*, about 1 to 0.3–0.4; in that of *Dendrocoelum lacteum*, according to the lower rate of carbohydrate metabolism, only 1 to 0.12–0.15. These figures demonstrate that *Planaria torva* uses during the first week of starvation about one-third, and *Dendrocoelum lacteum* about one-eighth, of the total oxygen in the carbohydrate metabolism. It is to be expected that in later stages of starvation the relative rate of carbohydrate metabolism will be reduced because the stores are almost exhausted after one week at 25° C.

SUMMARY

1. *Planaria torva* shows cyclical variations in the polysaccharide content.

2. The rate of carbohydrate metabolism decreases in *P. torva* during the single days of the first week of starvation at 25° C.

3. The rate of carbohydrate metabolism is higher in *P. torva* during the winter, when the worms have stored much polysaccharide, than during the summer, when the amount of polysaccharide stored is small.

4. The intensity of carbohydrate metabolism both in *P. torva* and *Dendrocoelum lacteum* is dependent, in high degree, upon the tem-

perature. The curve obtained for its increase with rising temperature does not conform to the van't Hoff curve but is much more like Krogh's normal curve.

5. Krogh's normal curve is an adequate expression for the relation of oxygen consumption to temperature in planarians.

6. *Planaria torva* uses during the winter about one-third of the total oxygen consumed in the carbohydrate metabolism; *Dendrocoelum lacteum*, only one-eighth.

7. In both species the proportion of carbohydrate metabolism to total metabolism remains the same during the single days of the first week of starvation.

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